EXHIBIT 6

HUMAN GENE TRANSFER/THERAPY PROTOCOL

9202-016

Freeman, Scott M., University of Rochester School of Medicine, Rochester, New York; *Gene Transfer for the Treatment of Cancer.*
Date of RAC Approval: 2/10/92

The Treatment of Ovarian Cancer with a Gene Modified Cancer Vaccine: A Phase I Study

University of Rochester Medical Center

INVESTIGATORS:

Scott Freeman, M.D. (P.I.)
Craig McCune, M.D.
George N. Abraham, M.D.
Cynthia Angel, M.D.
Martin Brower, M.D.
Susan Rosenthal, M.D.

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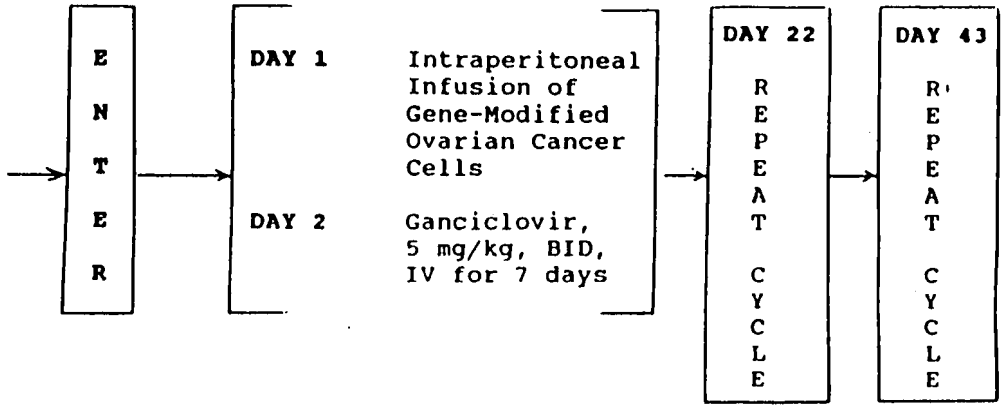
THE TREATMENT OF OVARIAN CANCER WITH A GENE-MODIFIED CANCER VACCINE: A PHASE I STUDY

Eligible

Stage I, II, or III Ovarian Cancer

Patients Who Have Completed Surgery and Chemotherapy

Evidence of Residual Disease by Tumor Marker or Imaging Must be Present



Goals

1) To evaluate the safety and side effects of treatment with a Gene-Modified Ovarian Cancer Vaccine which is administered intraperitoneally and activated by ganciclovir

2) To determine a maximum cell dose of the vaccine which can safely be administered intraperitoneally.

3) To evaluate the immunologic response to this vaccine program.

4) To observe for clinical effects on the residual ovarian cancer.

Gene-Modified Ovarian Cancer Vaccine

The Ovarian Carcinoma Cell Line, PA-1, has been transduced to express the Herpes Simplex Virus Thymidine Kinase, PA-1-STK.

Vaccine Dosage and Administration: A cell dose escalation plan will be followed with each patient such that a higher dose is given with repeat cycles. The assigned dose of cells will be prepared, radiated and administered through an intraperitoneal catheter in 1000 cc of normal saline.

URCC 2891M

Date																	
Time																	
Day		Tu	Th	F	F	Tu	Th	F	F	Tu	Th	F	F	Tu	Th	Tu	
Sequence	Pre	-2	1	2	10	20	22	23	30	41	43	45	52	62	64	83	
Vaccine			X				X				X						
Ganciclovir				X----X				X	X			X	X				
CBC, Plt Ct.	X					X				X				X			
12 Chemistry	X					X				X				X			
Creatinine Clearance	X																
CA-125	X					X				X				X			X
Skin Tests																X	
PA-1STK (gene-modified cell)		X					X				X						
Candida, SK/SD, Mumps		X				X				X				X			
Autologous Tumor Cells		X				X				X				X			
SK OV-3 (ovarian CA cells)		X					X				X					X	
Caki 2 (renal)		X				X				X				X			
Read Skin Test				X			X				X					X	
Cytotoxicity Assays		X				X								X			
Serum Sample		X				X								X			
Clinic Exam	X	X				X								X		X	
Imaging Measurement	X									X						X	
Laparoscopy/laparatomy	X																X

3.0 Introduction

3.1 Overall status of treatment of ovarian cancer

There are approximately 19,000 new cases of and 12,000 deaths from ovarian cancer each year (1). Mortality from ovarian cancer accounts for 6% of cancer deaths in females, and approximately 50% of gynecological malignancies (1). Ovarian cancer is staged into four catogories: Stage 1 - growth limited to ovaries, Stage II - growth involving one or more ovaries with pelvic extension, Stage III - tumor extending outside the pelvis and/or retroperitoneal or inguinal node involvement, and Stage IV - distant metastasis outside the peritoneal cavity. The first three stages can be divided into three substages of more progressive disease (a-b-c).

Patient survival can be evaluated based on the stage of the disease at diagnosis. Higher stage disease correlates with a worse prognosis. Treatment of ovarian cancer is based on the stage of the disease. Higher stage disease is treated with a more aggressive course of chemotherapy. Stage I disease can be treated with either surgery or surgery and chemotherapy. Stage II, III, and IV patients are initially surgically debulked of tumor before receiving chemotherapy and/or radiation therapy. The treatment is based on the ability to surgically remove the tumor and the location of the remaining tumor. The drug of choice to treat ovarian tumors is cisplatin. Patients in may be treated with intraperitoneal chemotherapy. Therefore, the technology allowing the insertion of fluid into the peritoneal cavity for treatment of ovarian cancer is established (1).

Ovarian cancer patient survival is: Stage 1 - 80-100%, Stage II to Stage IIIa - 30-40%, Stage IIIb - 20%, and Stage IIIc to IV - 5-10%. Ovarian cancer patient prognosis can also be estimated by either: 1) size of largest tumor lesion at the time of diagnosis, or 2) diameter of the largest remaining metastatic lesion after surgical reduction. Patients presenting with a >10 cm tumor mass exhibited a 0% three year survival and a 9 month mean survival (2). Patients with a >1.5 cm tumor mass post-surgical reduction exhibited a 0% three year survival with a 6 month mean survival (2). In addition, patients who relapse only have a 10-20% response to second line therapy which has not been shown to prolong patient survival (3).

The CA 125 marker has been useful in determining microscopic disease recurrence if the patient presented with a positive titer which decreased with treatment. CA 125 is a cell surface glycoprotein found on ovarian tumor cells. In a patient with ovarian cancer, a CA 125 titer of >35 IU/ml is almost always associated with disease. A negative titer is a poor indicator for lack of disease (4). The rising or falling of the CA 125 titer can be used to follow the success or failure of treatment. Thus, persistently elevated titers during treatment usually indicates treatment failure (4).

3.2 Progress in the investigation of vaccines for active-specific immunotherapy.

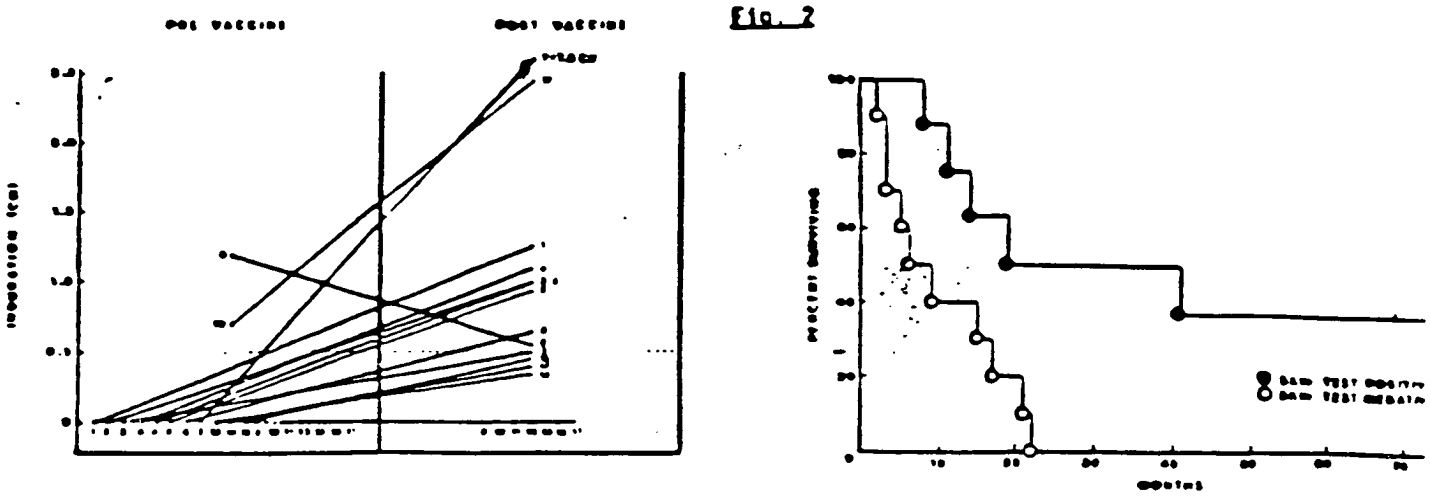
The clinical trials of vaccines for active-specific immunotherapy have had some success with three types of malignancy; renal carcinoma, colon carcinoma, and malignant melanoma. The methodologic requirements for cancer vaccines have gradually been worked out in animal models in the last 15 years. Many clinical trials in this field were initiated before this information was available, or did not adhere to the methodologic requirements that had been found in the animal studies. As a consequence, there are several clinical investigations of active immunotherapy failed due to study design or preparation of the tumor vaccine. These studies may have failed due to one of the following factors: (A) The tumor cells should be preserved in a viable form in those vaccines where whole tumor cells are used. (B) Each procedure toward a subcellular product must be carefully evaluated as the immunogenicity can be easily destroyed. (C) The cryopreservation procedure for strong tumor cells for a later use in the vaccine must be carefully evaluated and shown not to destroy the immunogenicity of the vaccine. (D) The use of an adjuvant is essential, but the adjuvant must be given at a predetermined optimal concentration. Tumor cell vaccines are considered to be weakly antigenic and the use of an effective adjuvant is critical to success, but selection of the wrong dose can negate the adjuvant's role. (E) The dose of tumor cells or quantity of antigenic material is a critical factor and when an inadequate dose is given, the immunologic effect will be lost. (F) The animal studies had predicted that the use of the patient's own tumor cells (autologous) would have the highest likelihood of success, while allogenic cells were often unsatisfactory. However, the use of allogeneic cell lines would have great practical advantages of standardization, availability, and ease of vaccine preparation.

Allogeneic approaches have begun to show promise in the treatment of malignant melanoma where some regressions of metastatic disease have been observed (5, 6) and preliminary but non-randomized studies for early melanoma have shown promise (7, 8).

One of us (C.M.) has been conducting trials on the concept of active-specific immunotherapy for metastatic kidney carcinomas using autologous tumor cells and C. parvum as an adjuvant. The vaccine was administered weekly for six weeks, 3 of 14 patients underwent objective regressions in the initial study (9), and 5 of 20 patients responded in a second trial (10). Several of the responding patients had remissions lasting from 2-5 years and some remain in remission. Skin testing was included by using autologous tumor cells for a portion of these renal carcinoma patients; the rationale was that if immunity to tumor cell membrane antigens is achieved, there should also be delayed cutaneous hypersensitivity on skin testing with those same tumor cells as had been observed in animal tumor models. Below are two figures summarizing our experiments with this group of renal carcinoma patients (11). In figure 1, the skin testing results shown for 18 patients who were tested prior to and following the treatment protocol. Eight of the eighteen patients became skin test positive patients, three had objective partial responses and the fourth had a minor

response followed by 47+ months of stable disease. Of the 10 skin test negative patients, none had a clinical response.

A survival outcome for skin test positive versus negative patients is shown in Figure 2. All the skin test negative patients had expired by 22 months while 4/8 skin test positive patients were alive at greater than 32 months. The survival times of these two groups differs with p = 0.03, by the generalized Wilcoxon (Breslow) test.



Clinical investigators have had some success with vaccines for advanced malignant melanoma. Berd, Maguire, and Mastrangelo (12) used autologous melanoma tumor cells for their vaccine with BCG as the adjuvant and have reported responses in melanoma patients in the 16-20% range with some responses being complete remissions which were long-lasting. Mitchell and associates (5) have been evaluating an allogeneic vaccine derived from two cultured melanoma lines and then prepared as a subcellular lysate. As an adjuvant they employed the agent Detox. They have observed responses in 20-30% of the patients in the Phase I and Phase II studies. Morton and associates have used a 3-line allogeneic melanoma vaccine and have obtained responses in 19% of their patients (6).

In colon cancer, Hanna and Hoover (13, 14) have conducted a prospective randomized study comparing surgical treatment only versus surgery followed by a vaccine of autologous tumor cells with BCG as the adjuvant. The survival outcome in these patients favored those receiving the vaccine at an early point when only 20 patients had been entered in each arm. The full study with about 75 patients participating continues to demonstrate a significant recurrence-free survival and overall survival for those patients treated with vaccines following surgery.

The study by Hanna and Hoover indicates the potential of the specific immunotherapy approach for the treatment of cancer. In order to attain a broader application for the treatment of cancer, we will need to move towards the use of a more

standardized vaccine such as can be achieved with allogeneic cell lines. Both the clinical experience by Mitchell (5) in melanoma and the animal studies employing allogeneic lines (15, 16) indicate that the use of allogeneic vaccines would be possible if there is sufficient diversity in the vaccines to represent a broad spectrum of antigens that can contribute to the immunogenicity of the vaccines.

3.3 Gene transfer clinical studies

"Gene Transfer and Therapy" protocols have been approved for human trials in the past few years (17). The initial Gene Transfer study was a gene marking study by Rosenberg and colleagues to mark tumor infiltrating lymphocytes (TIL) *in vitro* with the neo^R gene. These marked TIL were injected back into the patient in order to determine the *in vivo* fate of these cells. This was accomplished by using the polymerase chain reaction (PCR) technique to analyze cells isolated from the patient over time. These cultured TIL frequently demonstrate *in vitro* cytotoxicity to the tumor from which the cells were isolated and, cause necrosis of tumor deposits when injected into the patient (18). While melanoma patients have had up to a 10% complete response, the lack of significant response in the majority of treated patients has raised questions about the fate of infused TIL. Studies to determine cell survival have been limited by the instability of the radionucleotide used to label TIL. To avoid this limitation TIL cells were permanently marked with the LNL-6 retroviral vector, which contains the neomycin resistance gene as a marker (17). TIL transduced *in vitro* with the LNL-6 retroviral vector have been infused into patients and, subsequently, polymerase chain reaction (PCR) was used to detect the neo^R DNA sequence within a tissues (17). Retrovirally marked cells from 4 of 5 evaluable patients could be reisolated and grown in culture in the presence of G418, the neomycin analog toxic to mammalian cells. Using PCR, neo^R TIL could be consistently detected in the circulation for three to eight weeks post-infusion. The neo^R DNA could be detected by PCR in biopsies of patient tumor deposits for up to 64 days (17).

There have been two gene therapy protocols approved (19, 20). One protocol by Blaese and colleagues has genetically altered lymphocytes from adenosine deaminase deficiency patients. They have inserted the gene for adenosine deaminase (ADA), the defective gene in this disease, into these patients' lymphocytes. The transduced cells are grown to large numbers and then infused into the patient. The hope is that these cells with a normal ADA gene will also function normally and cure the immunodeficiency found in these patients. The second gene therapy protocol also involves the use of TIL. Clinical studies using recombinant TNF to infuse into patients have been unsuccessful possibly due to the toxic side effects of TNF. TNF producing TIL may be a more effective method to deliver the TNF to the tumor deposits. The TIL are genetically modified with a gene encoding the tumor necrosis factor (TNF) protein. These TNF producing TIL when injected into the patient may

migrate to the tumor, thus theoretically producing high local TNF levels while potentially avoiding the systemic side effects.

In addition, recently three human gene transfer protocols are being reviewed which serve to mark tumor cells in the bone marrow of patients undergoing autologous bone marrow transplantation (ABMT) (21). They will attempt to determine the role of these tumor cells on cancer relapse in these patients after ABMT. These protocols are the first to propose using retroviral stock on cells which will be immediately removed from the viral stock and injected into a patient, rather than first monitoring the cells *in vitro* for adverse side effects (i.e., replication competent virus production or tumor formation due to an oncogenic event). However, assays to detect replication competent virus will allow this procedure to be performed safely since these assays can detect replication competent virus in the viral stock.

The ability to transfer genes to non-transformed cells has become possible over the past decade. The most common method for gene transfer into non-transformed cells is by retroviral-mediated methods (22). Retroviral vectors, most commonly generated from the Moloney murine leukemia virus (M-MuLV), will efficiently transduce, stably insert into, and generate high levels of recombinant protein in host cells (23). The receptor for murine leukemia viruses is ubiquitous in mammalian species. A number of potential hazards exist when using the M-MuLV retroviral vector. First, the M-MuLV wild type retrovirus is a leukemia virus in some murine species. It can cause leukemia, in part, through protooncogene activation in the infected cell by the retroviral long terminal repeat (LTR). Second, though retroviral vectors are "crippled" retroviruses and thus are replication incompetent, they possess the ability to recombine with retroviruses within a packaging cell line to form a replication competent virus.

Though the M-MuLV retroviral vector still possesses its LTR, the frequency with which oncogenesis occurs in the transduced cells appears to be very low. The overwhelming majority of reported experiments performed over the past decade with retroviral vectors demonstrate no association with transformed cells. These studies include experiments in murine, dog, monkey, and sheep models to name a few. However, we describe below an association of retroviral vectors with transformed cells, which appears to be low in frequency.

The ability of retroviral vectors to undergo recombination events to produce replication competent virus can be detected by sensitive assays which have been established over the past decade. These assays are termed S^+/L^- and Helper Rescue Assay (24). If a recombination event occurs, it will almost always occur in the producer cell. The producer cell line contains both a helper virus and the retroviral vector. The producer cell line generates the viral particles used for transduction. Therefore, the producer cell line can be continually monitored for the appearance of replication competent virus. Cells transduced with replication incompetent viral stock, as

measured by the above assays, are very unlikely to generate replication competent virus themselves. However, as described in this protocol below, these transduced cells can also be monitored for a few weeks after transduction, a period sufficient to detect replication competent virus by the above assays. Thus monitoring transduced cells for a few weeks *in vitro* before injection into a patient will greatly reduce the risk of generating replication competent virus. This proposal will be designed such that the transduced ovarian tumor cells will be monitored for at least four weeks post-transduction before these cells are injected into a patient.

3.4 Preclinical studies

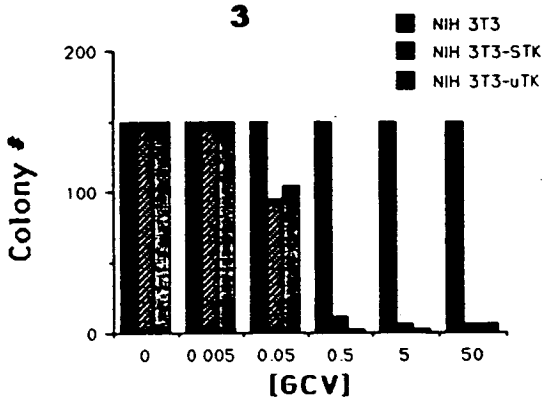
In vitro and *in vivo* studies

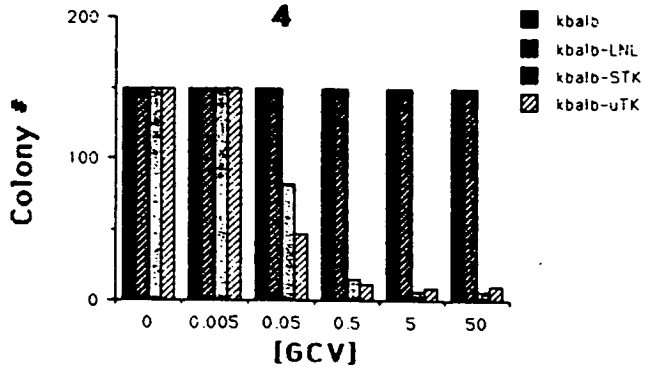
Tumor cells expressing the HSV-TK gene are killed *in vitro* and *in vivo* by the drug ganciclovir. HSV-TK expressing tumor cells can affect nearby HSV-TK negative tumor cells when exposed to GCV. The ability to kill tumor cells *in vivo* may affect preexisting tumor cells in two ways: 1) transfer of the HSV-TK phenotype from the HSV-TK positive cells to the HSV-TK negative cells, thus rendering the HSV-TK cells susceptible to ganciclovir therapy and 2) generation of host immunity against the killed tumor cells. The studies presented in this section will address both issues, with the main focus being on the former.

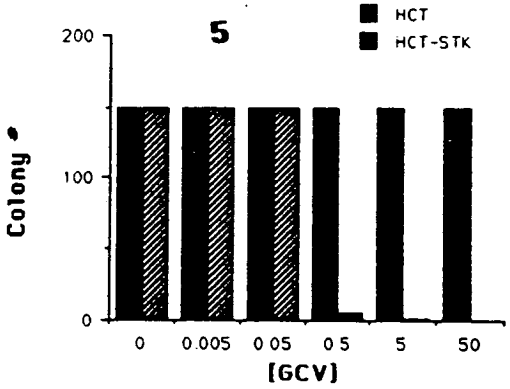
Initial studies analyzed the ability of HSV-TK transduced tumor cells to respond to ganciclovir (GCV) *in vitro*. A murine fibroblast cell line, NIH 3T3, was transduced with either the STK or uTK, a neo^R and HSV-TK containing retorviral vectors (25). These transduced cells were selected in G418, a neomycin analog toxic to mammalian cells. A control population of NIH 3T3 cells was transduced with the LNL retroviral vector(provided by Dr. A.D. Miller), a neo^R containing retroviral vector, and selected in G418. The cells were then placed in varying concentrations of GCV for 10-14 days at which time live cells (colonies) were counted. Cells expressing the herpes simplex thymidine kinase gene (HSV-TK) are susceptible to the drug ganciclovir (GCV). GCV is a nucleotide analog which is phosphorylated by HSV-TK with a 2-3 log efficiency as compared to the cellular thymidine kinase. The phosphorylated compound can then be di- and triphosphorylated by cellular enzymes. The triphosphate form of GCV is toxic to the cell by either functioning as a DNA polymerase inhibitor or a DNA chain terminator or both. There is no obvious variation between the ability of GCV to kill either the STK or uTK transduced cells, while control cells were not affected by GCV (figure 3).

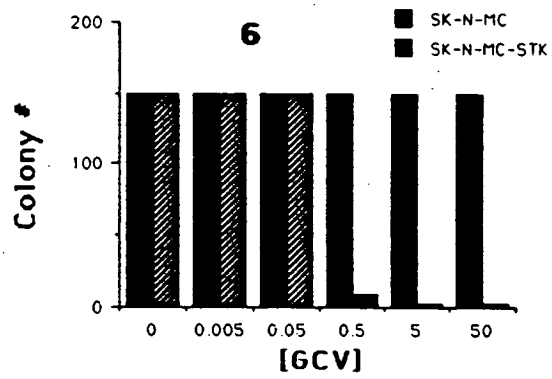
We next transduced a murine fibrosarcoma cell line, kbalb, with the STK, uTK, and LNL retroviral vector. Almost all HSV-TK positive cells were killed by >0.5 uM GCV, while the untransduced or LNL transduced kbalb cells were virtually unaffected at GCV concentrations of 50 uM (figure 4). The most colonies which could be counted on a plate is 150. Therefore, confluent plates which contain too many colonies to count will be scored as 150 colonies. These studies were repeated with human tumor cell lines, SK-N-SH and HCT (neuroblastoma and colon carcinoma), with the same results

(figures 5 and 6). Of note is the approximately 1% GCV resistant colonies in some instances observed at high GCV concentrations. The significance of these cells will be discussed below.

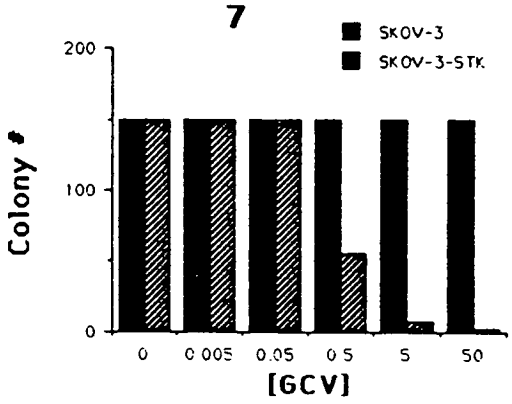


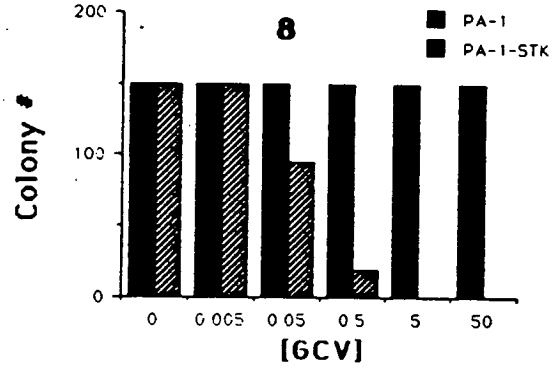




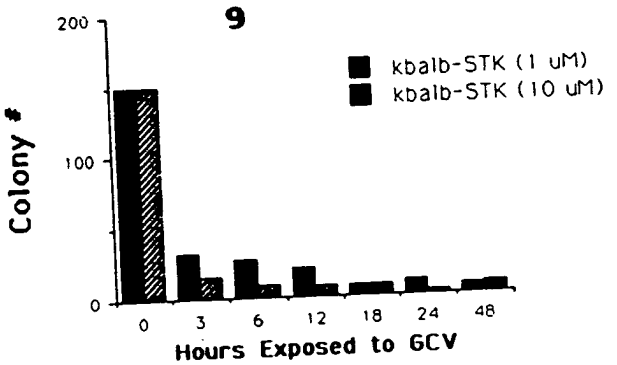


In addition, two ovarian carcinoma cell lines, SKOV-3 and PA-1, were transduced with the STK retroviral vector and exposed to varying concentrations of ganciclovir. Figures 7 and 8 demonstrate a similar cell toxicity as is seen in experiments above.



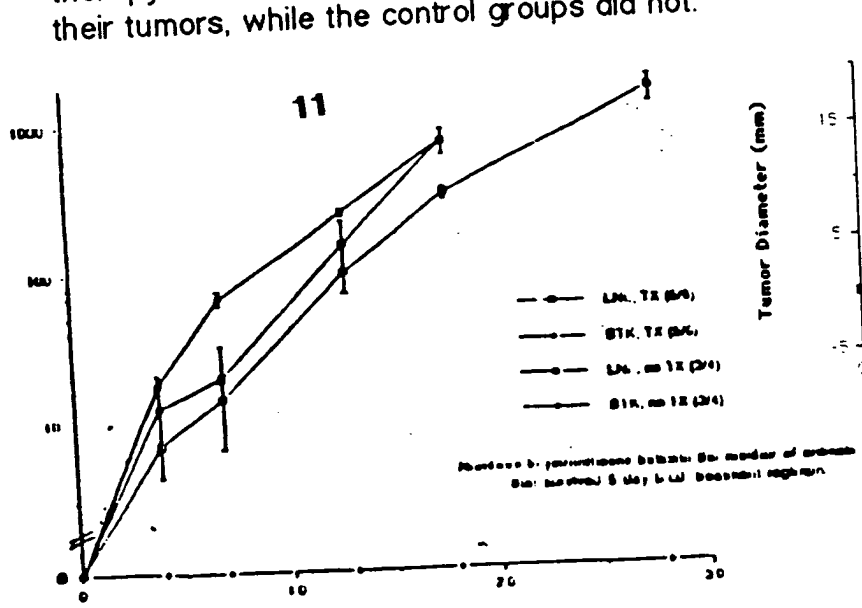


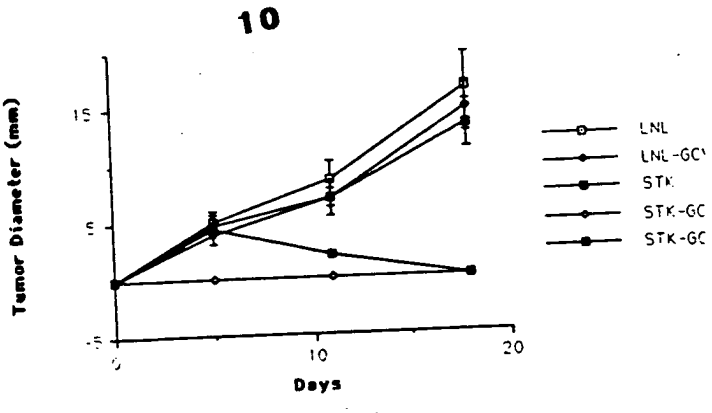
We next studied the time kinetics of cell kill of TK positive cells by ganciclovir. Using either 10 uM or 1 uM GCV, the approximate peak and trough levels achieved *in vivo*, respectively, kbalb-STK cells were exposed over varying time periods to these GCV concentrations. We demonstrated that exposure of kbalb-STK cells to 10 uM GCV for only three hours resulted in almost 100% killing of the cell population (figure 9).



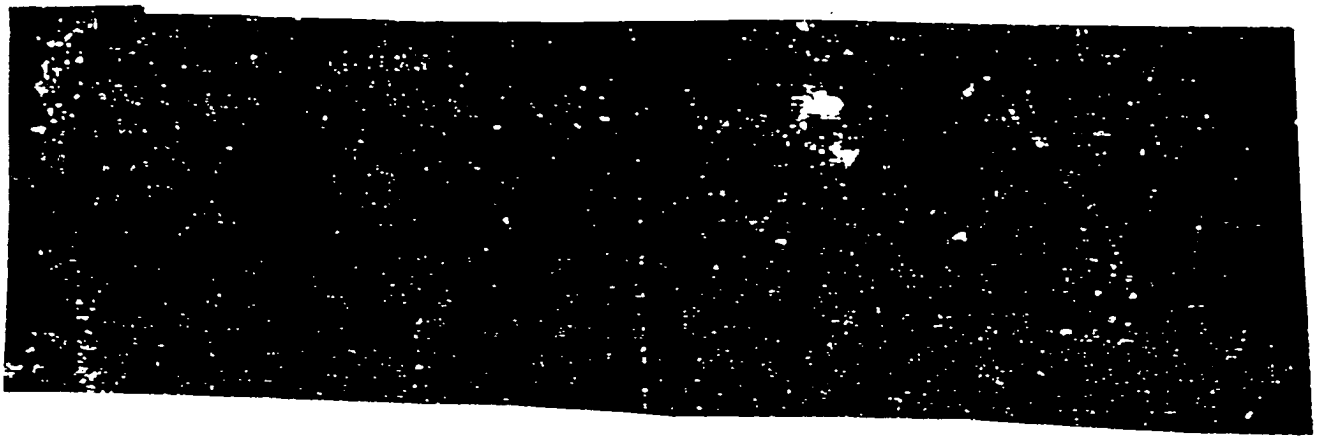
Initial observations described above demonstrated that some STK transduced cells were resistant to GCV therapy *in vitro*. It was surprising to find that an HSV-TK (TK) positive cell population could be eradicated *in vivo* even if enough cells were inoculated to assure adequate numbers of GCV resistant tumor cells to form a tumor.

The ability to kill TK positive tumor cells *in vivo* was next addressed. The kbalb-STK and control LNL transduced kbalb cells (figure 10), 2 x 10^5, as well as three other murine tumors (SA-1 (figure 11), MC 207, and MC 205) were inoculated subcutaneously into syngeneic mice. Mice were treated with ganciclovir (150 mg/kg/dose x 5-10 doses) beginning on day 0 or day 5 relative to time of tumor inoculation. Some animals received kbalb-STK or kbalb-LNL cells without GCV therapy. Mice which received STK transduced tumor cells and GCV therapy rejected their tumors, while the control groups did not.





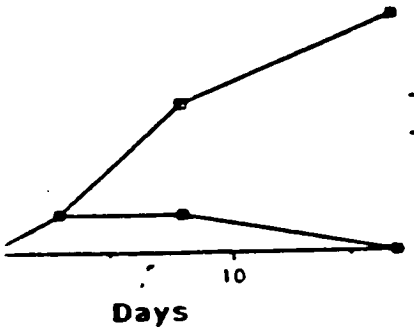
Studies on the possible mechanism for the affect of TK positive cells on TK negative cells were begun by analyzing the mechanism of cell death when TK positive cells are exposed to GCV. Cells die by two mechanisms, necrosis and apoptosis. Necrotic cell death is characterized by cell swelling, cell membrane disintegration, and nuclear flocculation. Apoptotic cell death is characterized by cell shrinkage, vesicle formation, and chromatin condensation. Cells dying by apoptosis break up into vesicles which can be phagocytized by nearby cells. Tumor cells have the ability to phagocytize apoptotic vesicles. TK negative cells may be affected by TK positive cells due to the ability of TK negative cells to phagocytize toxic metabolites contained in TK positive apoptotic vesicles. Plates 1 and 2 show light microscopy of normal and TK positive cells exposed GCV. The TK positive cells show the characteristic signs of apoptosis: cell shrinkage, vesicle formation, and chromatin condensation.



Tumor Diameter (mm)

We next analyzed the effects of TK positive cells on TK negative cells *in vivo*. Using the kbalb tumor model, mice were inoculated with varying ratios of kbalb tumor cells which were transduced with either the STK or LNL retroviral vector. Mice received 2 x 10^5 tumor cells subcutaneously on day 0 and were treated with GCV on day 5 when tumor diameter was approximately 2 mm (150 mg/kg I.P., b.i.d., x 10 doses). Groups of mice receiving either 50%, 90% or 100% kbalb-STK cells demonstrated tumor regression (figure 15). These studies were repeated with two other tumor lines, 205 and 207. In these experiments untransduced tumor cells were mixed with STK transduced tumor cells in varying ratios of 0%, 1%, 10%, 50%, 90% and 100% 205-STK or 207-STK. All animals in groups receiving 50% or more STK transduced cells demonstrated tumor regression. However, some animals receiving 10% STK transduced cells developed tumors as did all animals in groups receiving 1% or 0% STK transduced cells (figure 16).

15



16

% STK	# Mice	% Rejection
100	5	100
50	5	100
10	4	75
1	4	0
0	5	0

The ability of immunodeficient mice to reject STK transduced cells was also studied. Nude and non-lethally irradiated mice were inoculated subcutaneously with 205-STK or 207-STK tumor cells at varying ratios as described above and treated with GCV. The immunodeficient mice did not reject tumor even when 100% of the tumor cells contained the HSV-TK gene (figure 17 and 18). Therefore, there appears to be possibly two mechanisms involved in the killing effect of TK positive cells on TK negative cells.

17

Non-lethally Irradiated

% STK	# Mice	% Rejection
100	4	25
50	4	0
0	3	0

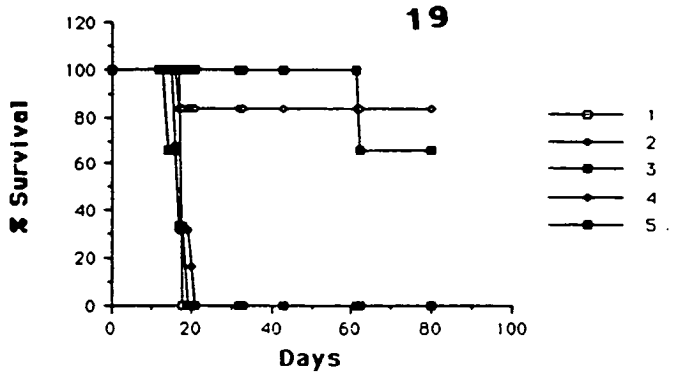
18

Nude

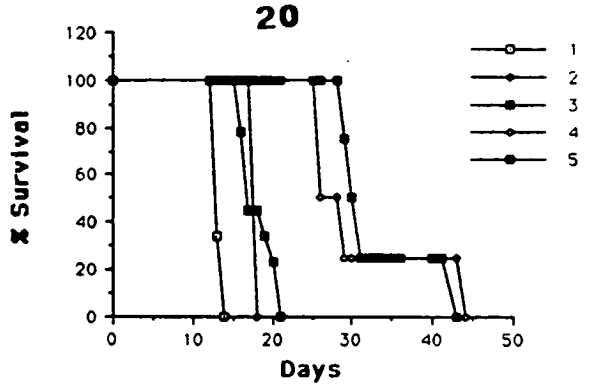
% STK	# Mice	% Reject.
100	4	25
50	3	0
0	3	0

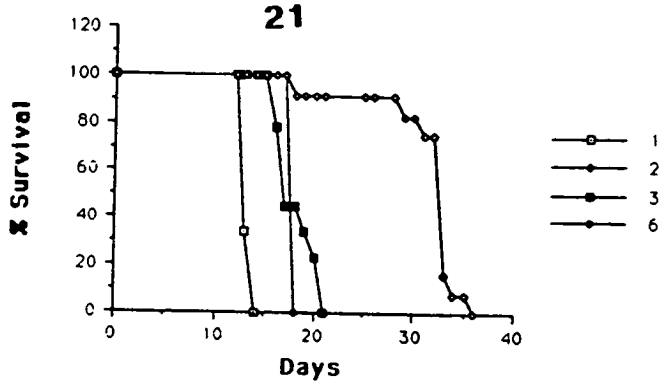
The effect of kbalb TK positive tumor cells on TK negative cells was analyzed using an intraperitoneal tumor model. Previous experiments demonstrated that in a relatively confined space, subcutaneously, as few as 50% TK positive cells within a tumor could cause regression of the tumor. Varying ratios of kbalb tumor cells ($2x10^5$) transduced with either the STK or LNL retroviral vector were injected I.P. into Balb/c

mice. Mice were treated with GCV (150 mg/kg I.P., b.i.d., x 5 doses) five days post tumor inoculation. Survival studies were then performed. Groups of mice receiving either 50% or 100% STK transduced tumor cells contained mice which exhibited long term survival (> 70 days), Groups 5 and 4 respectively (figure 19). Control mice, receiving kbalb-LNL cells +/- GCV or kbalb-STK without GCV did not exhibit long term survival (Groups 1, 2, and 3). Groups receiving 1% or 10% STK transduced cells contained no long term survivors but had a trend towards surviving longer than the controls (data not shown). Therefore, the ability of TK positive tumor cells to affect TK negative tumor cells can be demonstrated in a large enclosed body cavity.



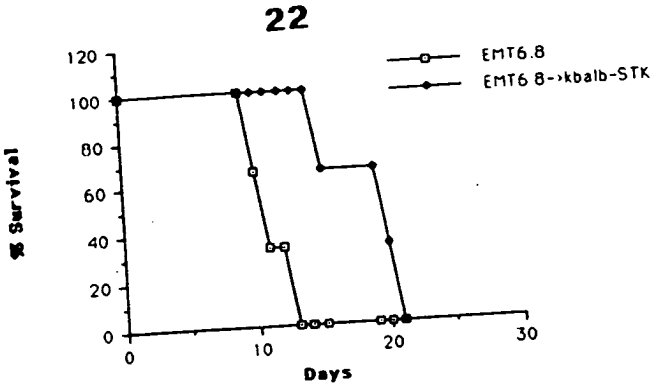
We next analyzed whether TK positive tumor cells could affect a preexisting tumor. The kbalb tumor model was used to address this question. Mice were inoculated I.P. with 2 x 10^5 kbalb-LNL (except Group 1 which received kbalb-STK) tumor cells on day 0. Group 1 and 2 received no further treatment. Group 3 received GCV therapy beginning on day 5. Group 4 received 1 x 10^6 kbalb-STK cells and Group 5 received 1 x 10^7 kbalb-STK cells on day 5 and GCV beginning on day 9. Group 6 mice received 1 x 10^6 kbalb-STK cells on day 1 and GCV therapy beginning on day 5. As shown in figures 20 and 21, mice in Groups 4, 5, and 6 had prolonged survival as compared to control groups (Groups 1,2, and 3). Group 6 mice had a mean survival of approximately 31 days as compared to 18 days for Group 3 ($p < 0.05$).

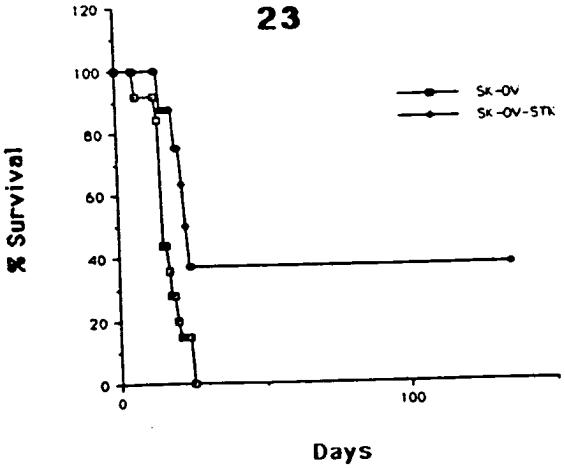




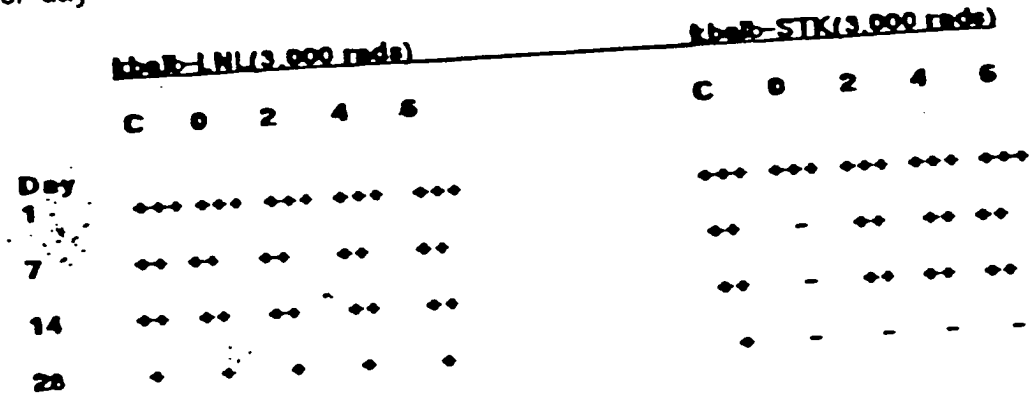
The above experiment which demonstrated the effect of kbalb-STK transduced tumor cells on preexisting tumor was repeated using other tumor lines. On day 0 mice were inoculated with EMT 6.8 tumor cells (murine mammary tumor) alone (Group 1) or

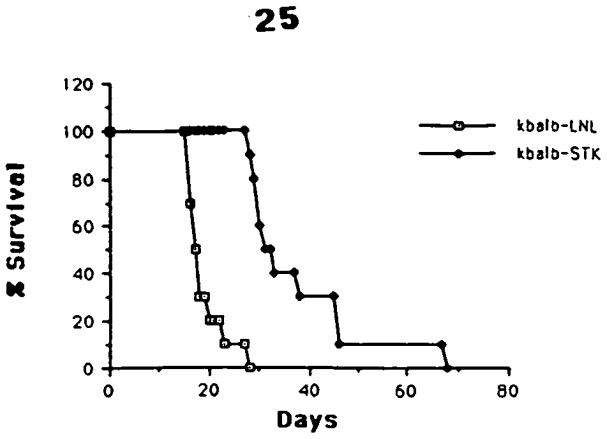
received on day 1 kbalb-STK cells. Both groups received GCV on day 5 for 5 doses (figure 22). Another experiment used the SKOV-3 tumor cell line as the day 1 inoculum. Mice injected on day 0 with kbalb-LNL tumor cells either received no further cells or 2 x 10^6 SKOV-3 (Group 1) or 2 x 10^6 SKOV-3-STK tumor cells on day 1 with GCV therapy beginning on day 4 for 7 doses (Group 2)(figure 23). Group 2 was the only group of mice with a preexisting tumor burden to have long term survivors in any experiment. It also appears that injection of xenogeneic tumor cells does not affect tumor growth of a preexisting murine tumor.

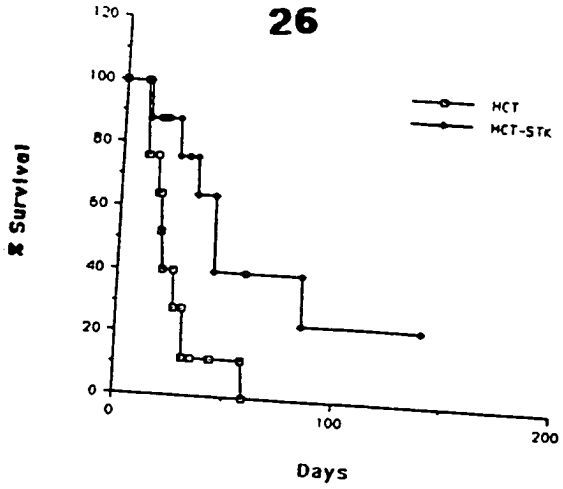




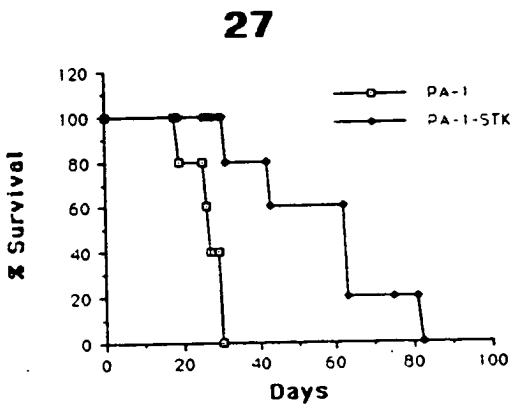
The ability of GCV to kill irradiated HSV-TK expressing tumor cells was analyzed. The kbalb-LNL or kbalb-STK tumor cells were exposed to 3,000 rads and then cultured *in vitro*. The cells were exposed to 50 uM GCV at varying times post-irradiation ranging from no GCV (Group C) to GCV added on day 0, day 2, day 4, or day 6 post irradiation (Group 0, 2, 4, and 6 respectively). The irradiated kblab-LNL tumor cells exposed to GCV continued to remain metabolically active as evidenced by their ability to remain attached to a tissue culture dish up to 28 days post irradiation (the left column of Table I represents the days on which the irradiated cells were observed for metabolically active cells). The attached cells were qualitatively scored; ++++ indicates approximately 90-100% of the plate is covered by cells, +++ indicates 50-90% of the dish is covered, ++ indicates 10-50% of the dish is covered, + indicates <10% of the dish covered with cells, and - indicates <1% covered with cells. We determined that GCV had virtually no affect on irradiated TK negative cells. Irradiated TK positive cells exposed to GCV on day 0 died within a week, while cells exposed to GCV after day 0 died after approximately one month (Table I).

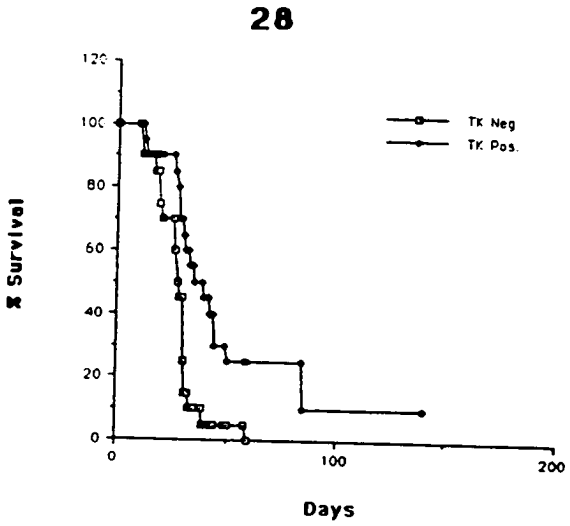






Irradiated PA-1 cells were assayed for their ability to prolong animal survival in mice with preexisting tumor burden. PA-1 cells were transduced with the STK retroviral vector. The same experimental design was used as with the HCT-STK cells described in figure 26. Figure 27 demonstrates that PA-1-STK cells will also prolong the survival of mice with a preexisting kbalb intraperitoneal tumor. Figure 28 shows the results of a a combination of results obtained in figure 26 and 27 and a repeat experiment using PA-1-STK cells.





Immunization studies

A future goal of this protocol is for the patient to be immunized against their ovarian tumor. This may be possible in two ways. First, the killing effect of TK positive cells on TK negative cells may generate an immune response to the patient's own ovarian carcinoma. Second, one of us (C.M.) is studying the use of adjuvants on the ability to vaccinate colon carcinoma patients to their tumor by using an allogeneic tumor cell vaccine in conjunction with IL-1 beta. Therefore, in the future, adjuvants may be a possible method added to this protocol to increase the ability of the dying irradiated TK positive tumor cells to also function as a vaccine.

To study the effect of killing STK transduced cells *in vivo* mice were subcutaneously

In vitro mixing experiments were set up using irradiated TK positive cells (HCT-STK, human colon carcinoma) and non-irradiated TK negative cells (kbalb-LNL). Cells were plated at varying ratios of irradiated TK positive to TK negative cells with a total of 2×10^6 cells plated in a 100mm culture dish and GCV (50 uM) was immediately added. The results approximate those seen in the above experiments using non-irradiate TK positive tumor cells. When the TK positive cells made up as few as 10% of the cells virtually all the irradiated HCT-STK and non-irradiated kbalb-LNL cells were killed by GCV, while irradiated HCT tumor cells had no effect on the kbalb-LNL cells (figure 24).

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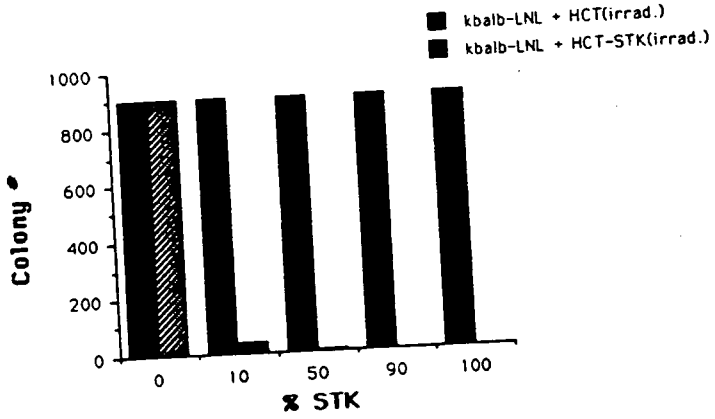


We next determined the affect of irradiated murine and human tumors on mice with preexisting murine (kbalb) tumor. Mice received 2×10^5 kbalb-LNL tumor cells on day 0 and 5×10^6 kbalb-LNL or kbalb-STK cells on day 1 and day 2. GCV therapy was begun on day 3 (7 doses). Figure 25 shows results which demonstrate that injection of irradiated kbalb-STK cells approximate those seen using non-irradiated kbalb-STK cells. The mean survival was 19 days for the controls and 38 days for the mice receiving TK positive cells ($p < 0.05$). We also repeated the experiment by using the HCT colon carcinoma cell line. 2×10^5 kbalb-LNL cells were injected on day 0 and 2×10^7 HCT or HCT-STK cells were injected on day 2 followed by 7 doses of GCV beginning 12 hours later. We demonstrated long term survival (> 70 days) in approximately 25% (figure 26).

combined controls. Five of eight experimental mice demonstrated no tumor growth at 21 days post challenge, though one animal subsequently developed a tumor. One of eight animal's tumor challenge grew slower than the controls, while two experimental mice grew tumors at similar rates as the controls. Table II shows the results of both the kbalb and 205 tumor models expressed as a percentage of tumor rejection after rechallenge of mice which had rejected a TK positive tumor with approximately 50% of the mice rejecting tumor rechallenges.

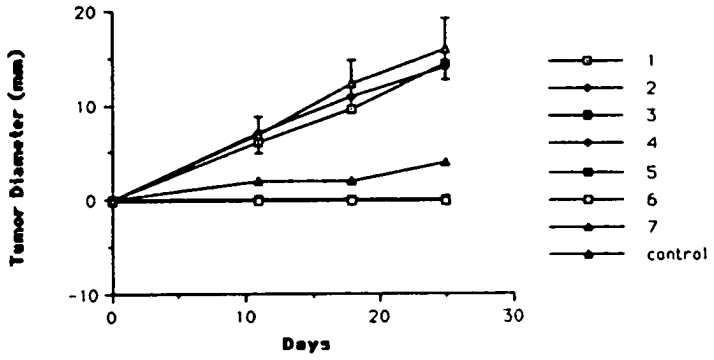


Table II

Tumor	Total	Reject.	% Reject.
kbalb	8 mice	4 mice	50
205	28 mice	13 mice	44

IL-1 studies *in vitro* indicate that this molecule is critical in the process of antigen activation of T cells. The activated T cells can then produce the spectrum of regulatory cytokines which lead to antibody production and cellular immunity. It appears then that IL-1 is a cytokine which acts early in the cascade of events leading to an immune response, and it is therefore attractive to explore the use of this cytokine in an adjuvant role to enhance the immune response. It has been found that several adjuvants function through production of IL-1. The agents lentinan (26), lipopolysaccharide (27), aluminum hydroxide (28), Ru 41.740 (29) (extract of k. pneumoniae), and tuftsin (13)

have all been found to increase IL-1 production or are thought for other reasons to function by means of IL-1.

Early studies of IL-1 by Staruch and Wood (31) demonstrated an increase in the antibody response to bovine serum albumin in mice from 4-fold to 30-fold above the use of BSA alone. A single intraperitoneal injection could achieve this effect.

We have investigated the potential adjuvant function of IL-1 in a murine lung cancer tumor system (32) which uses a weakly immunogenic tumor cell vaccine of line 1 tumor. We evaluated human IL-1 alpha, recombinant human IL-1 beta, and the peptide fragment 163-171 of IL-1 beta. All were tested in combination with a vaccine of irradiated tumor cells. All three types of IL-1 molecules were capable of improving the vaccine's effectiveness. The benefit of IL-1 was both dose dependent and duration dependent (the number of daily doses given). IL-1 functioned as a systemic adjuvant; that is, it was effective even when administered at a site distant from the vaccine. It was required that the IL-1 be administered during the 10 day period following the vaccine rather than being given at some point later. In this tumor model, mice receiving vaccine alone were only 0-20% tumor-free at the conclusion of the experiments. When eight daily doses of IL-1 were given along with the vaccine, 70-100% of the mice became tumor-free. An illustrative experiment from this work is shown in Table III. Both doses of IL-1 beta were highly effective as adjuvants given with the vaccine. These animals were weighed at the beginning and at the end of the 8 day period of treatment with IL-1. The higher dose of IL-1 effected weight gain, probably by virtue of the anorexia known to occur with high doses of IL-1 (33). However, the lower dose of IL-1 was well tolerated without an effect on weight gain and continued to be highly effective.

The Adjuvant Effect of IL-1β

Group	Vaccine	IL-1β(ng)	Mean Survival (Days ± S.E.)[a]	Tumor Free/ Total (%)[a]
1	-	-	14.90 ± 1.02	0/10 (0)
2	$3X10^5$ TC	-	17.00 ± 0.97	0/10 (0)
3	$3X10^5$ TC	120 d1-8	33.80 ± 3.66[b]	7/10 (70)[c]
4	$3X10^5$ TC	360 d1-8	40.00 ± 0.00[b]	10/10 (100)[d]

[a] Statistical comparisons with group 2 are indicated.

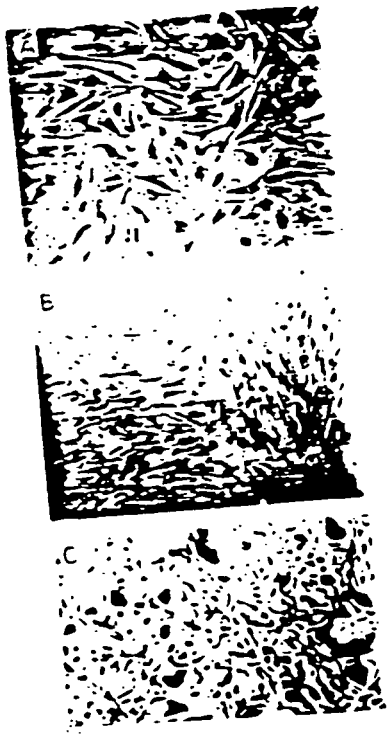
[b] $p < .001$ [c] $p = .001$, [d] $p < .0004$

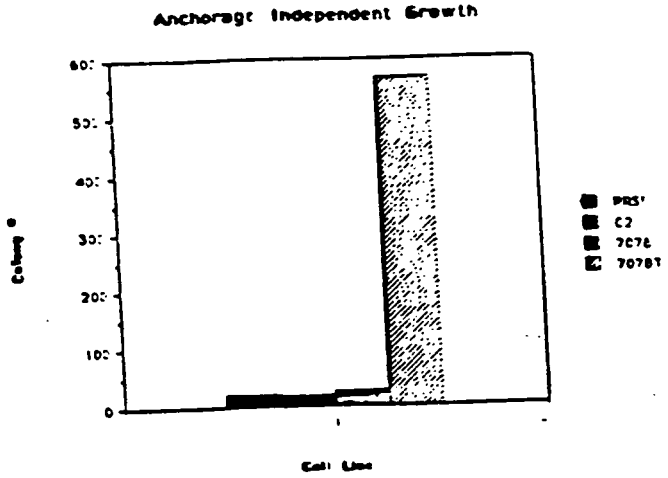
Safety

To address safety issues three areas were evaluated; 1) retroviral insertional oncogenesis, 2) *in vitro* studies , and 3) safety studies based on experience from the human clinical trials.

Retroviral *In Vivo* Studies

The following experimental data does not relate to the STK retroviral vector, it does relate to the effects of retroviral transduction on oncogenesis and therefore will be presented in this context. Studies were undertaken to analyze the effects of *in vivo* passage on retrovirally transduced cells. Rat skin fibroblasts were isolated from a Fischer 344 rat and expanded in tissue culture. The non-immortalized cell line was designated PRSF and transduced with the pG2N retroviral vector. This vector is an N2 based vector with an LTR (long terminal repeat) promoted growth hormone cDNA and a SV40 promoted neomycin resistance gene (neoR). The transduced PRSF cells were selected in G418 for 14 days and subsequently single clones were isolated. One clone, C2, producing approximately 600 ng/106 cells/ 24^0 was isolated. The C2 population of cells was used for implantation into hypophysectomized Fischer 344 rats. Alginate, a mucopolysaccharide, was used to encapsulate the C2 cells prior to implantation and the cell alginate mixture was implanted intraperitoneally (I.P.). We had previously demonstrated that encapsulated cells survived and secreted growth hormone for up to two weeks in tissue culture. Nineteen days post-implantation at a time when serum growth hormone levels were undetectable, one rat was sacrificed and the I.P. contents, consisting of a slurry mixture, were isolated and grown in tissue culture in the presence of G418. Two cell populations were isolated as distinguished by morphology as depicted in figure 26. Plate A shows the original pre-implantation C2 cells as compared to plate B, which was one isolated cell population (7078) exhibiting normal morphology, and plate C, a second population (7078T) which was spindly in appearance and grows in low serum (figure 30). The C2, 7078, and 7078T cells were all G418 resistant and produced growth hormone at approximately 600 ng/106 cells/24^0. The four cell populations , PRSF, C2, 7078, and 7078T, were plated in soft agar to assay for transformation. Virtually no colonies formed in agar from any of the cell populations except the 7078T cells, which had a plating efficiency of 2.8% (figure 31). In addition, colony formation of the 7078T cells demonstrated a larger colony size than the colonies formed by the non-transformed lines.These cell lines





were also inoculated into nude mice with only the 7078T cells forming tumors. Therefore, the morphology and ability to grow in low serum, as well as, anchorage independent growth and tumor formation in nude mice indicates that the 7078T cell line was transformed.

We next performed Southern blot analysis and showed that the pG2N retroviral vector was present in the C2, 7078, and 7078T cell lines (figure 32). The clonality of C2 and 7078 can also be demonstrated by Bgl II restriction enzyme digests, an enzyme that digests within the retroviral vector, by the presence of a single identical band in the C2 and 7078 cell lines. Finally, we can show not only the rearrangement of the growth hormone gene in the 7078T cells but also the rearrangement the c-myc oncogene (figure 33). Since the G2N retroviral vector did not insert near the c-myc oncogene, it may be possible that the G2N vector produces a trans activating factor which effected the c-myc oncogene. This represent the first reported association of a retroviral vector and a transformed cell.

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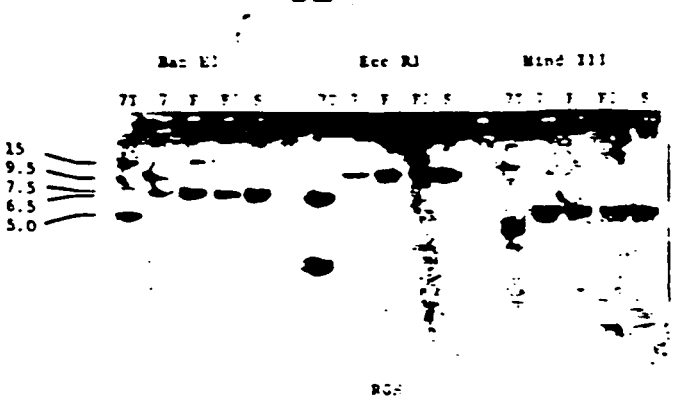

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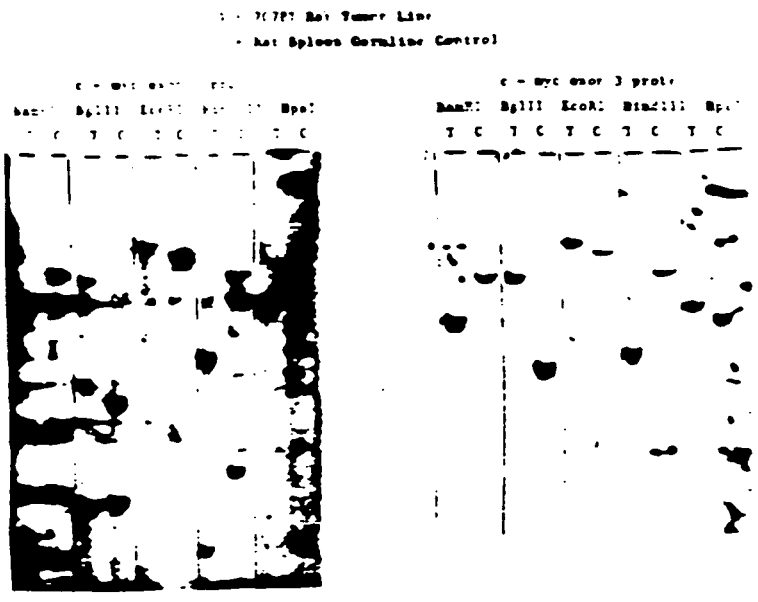

In Vitro Studies

We have obtained preliminary data in a mammary cell line that the NGW retroviral vector can transform these cells *in vitro*, as determined by anchorage independent growth. The NGW retroviral vector contains traditionally non-oncogenic genes (growth hormone and neo^R) and promoters. We are currently investigating whether the genes contained within the retroviral vector or the viral LTR's are responsible for the transformation process.

Non-Human Primate and Human Safety Studies

A non-human primate model was established by Cornetta et al (34) to analyze the affect of amphotropic retrovirus on monkeys. Five normal or immunosuppressed monkeys were inoculated with amphotropic viral stock to determine adverse effects of amphotropic virus in monkeys. In addition, some monkeys also received virus producing fibroblasts. Some immunosuppressed monkeys became viremic for approximately three weeks, but subsequently recovered with no repeat episodes of viremia or adverse effects.

To date, approximately one dozen patients have received genetically engineered cells with no adverse effects (17, 19, and 20). The initial studies by Rosenberg et al. injected genetically marked TIL cells into cancer patients. These cells were transduced with replication incompetent viral stock. The viral stock was monitored for the presence of replication competent virus, a by product of a recombination event, by the sensitive S+/L- assay or helper rescue assay. In addition, once transduced, the TIL cells were grown *in vitro* for almost a month and tested for replication competent virus in the event that a replication competent viral particle went undetected in the viral stock. Growing the cells in culture for a few weeks prior to reinfusion also allows the opportunity to analyze the cells for an oncogenic event produced by the insertion of the retroviral vector near a protoncogene. The next two human gene transfer clinical protocols followed similar procedures.

Recently, clinical protocols have been submitted which transduce and infuse bone marrow cells into a patient within a few days of the transduction process (21). These protocols which for technical reasons cannot monitor the transduced cells for replication competent virus or an oncogenic event before reinjection are still considered safe since the viral stock is tested by the above described sensitive techniques. In addition, the frequency of oncogenesis using murine retroviral vectors is very low. These factors are considered before an approval of the protocol in the context of the risk-benefit to the cancer patient. In the advanced cancer patients used in the last studies, the inability to monitor transduced cells long term before reinfusion was deemed an acceptable risk.

Our protocol also uses advanced cancer patients, but we will be able to monitor the

transduced cells at least four weeks post transduction to assay for the presence of replication competent virus before reinfusion of the cells into the patients. In addition, we plan to irradiate the cells prior to injection and thus will not assay for oncogenic events.

Murine Safety Studies

The kbalb-STK and PA-1 STK cell lines are being tested for safety by inoculation into Balb/c mice. The kbalb-STK (Group I) cell was irradiated and then 2 x 107 tumor cells were inoculated I.P.. Mice have been observed for 30 days with no evidence of adverse effects. The PA-1 STK cell line was irradiated and 2 x 107 cells were inoculated I.P. (Group II). These mice received ganciclovir therapy. This procedure has been repeated a second time in the mice, three weeks after the first injection, with no evidence of adverse effects 30 days post initiation of therapy (Table III).

Table III

Group	# of mice	RX	Days	Adverse Effects
I	20	-	30	-
II	10	GCV	30	-

4.0 Objectives of the Study

4.1 To evaluate the safety and side effects of treatment with a Gene-Modified Ovarian Cancer Vaccine which is administered intraperitoneally and activated with ganciclovir.

4.2 To determine a maximum cell number of the vaccine which can safely be administered intraperitoneally.

4.3 To evaluate the immunologic response to this vaccine program.

4.4 To observe for clinical effects on the residual ovarian cancer.

5.0 Patient Selection

5.1 A diagnosis of epithelial ovarian carcinoma must be established histologically.

5.2 Patients may initially have been Stage I, II, or III. Stage IV patients initially or at present are not eligible.

5.3 Patients must have completed the initial surgery and/or chemotherapy and have been off treatment for at least 6 weeks. Previous chemotherapy must have included cisplatin or carboplatin containing regimens.

5.4 Patients must have clinical evidence of recurrent, progressive or residual disease by imaging, physical examination, surgery, or successive elevation of the CA 125 marker. If possible, persistence or progression should be documented by histology/cytology. If CA 125 is used as evidence of residual disease, then the level must be greater than 35 IU/ml and increasing on two successive determinations, greater than 1 month apart, and the patient must not have had a laporotomy within the preceding 3 months.

5.5 The patients performance status must be 0 or 1 by ECOG standards.

5.6 Any tumor masses found by imaging must be 2.0 cm or less to be eligible for this study.

5.7 Adequate bone marrow, kidney and liver function must be shown by: Hct > 30, WBC > 4,000, platelets > 100,000, creatinine < 1.5 mg%, Creat. Cl > 50, normal bilirubin, SGOT and alkaline phosphatase < 1.5 x normal.

5.8 Patients must not have a significant history of heart disease (frequent angina, MI within the past 6 mos., congestive heart failure requiring daily treatment).

5.9 A patient is not eligible if there is a history of a previous malignancy, other than squamous or basal cell carcinoma of the skin. Patients with childbearing potential are not eligible.

6.0 Study Design

This is a phase I study to determine the toxicity and safety of administering HSV-TK modified ovarian tumor cells I.P with subsequent ganciclovir therapy. The

maximum tolerated dose (MTD) (up to 1 x 10^{10} cells per dose) of genetically modified tumor cells will be determined. If the MTD has not been determined by four groups of patients, an additional group could be added at an appropriately higher dose. Four patients will initially receive 3 x 10^7 HSV-TK positive tumor cells I.P. on day 0. Approximately twenty-four hours after the I.P. infusion the patient will be started on a seven day course of ganciclovir. Two weeks after the termination of ganciclovir therapy the patient will be reevaluated in terms of side effects from treatment and disease state. If no adverse toxicity occurred from the previous I.P. tumor injection and ganciclovir therapy and there appears to be no evidence of progressing tumor which would require chemotherapy, then the patient will be eligible for the next dose. This schedule will continue for doses of 1 x 10^8, 3 x 10^8 for Group 1. If no adverse side effects warranting the stoppage of therapy are reported in Group 1 then Group 2 patients will at one dose level higher as shown in sec. 8.2 and this pattern will continue through the 4 groups. If a patient has to drop out of the study for reasons other than toxicity, then another patient may be added to the group and will begin therapy at the lowest dose for that group. By entering patients at each dose level, we shall attempt to address the question of the maximum tolerated dose (MTD).

7.0 Patient Registration

Patients may enter this study by completing a consent form. Their eligibility will be confirmed by the data manager at the University of Rochester Cancer Center. Patient's will be assigned to Groups by the data manager. Participation or non- participation in this clinical study will not affect other therapy for which the patient is eligible.

8.0 Treatment Plan

8.1 The patent's will be admitted to the hospital for the first eight days of each cycle. The vaccine is given on day 1 and ganciclovir on days 2-8. The treatment is repeated at 3 week intervals for a total of 3 cycles of treatment. At the completion of treatment, patients will be followed regularly until their is progression of disease.

8.2 Vaccine and administration. Patients will be assigned in order of entrance on study to one of the four treatment schedules below. The dose escalates with each treatment unless any toxicity of grade II or higher level has occurred (Appendix B). For Grade II toxicity (except for neutropenia or thrombocytopenia) the dose is repeated but physician

discretion could lower the dose if overall toxicity is of concern. For Grade III or IV toxicity, the dose is lowered one level.

Patients	first dose	second dose	third dose
1-4	3×10^7	1×10^8	3×10^8
5-8	1×10^8	3×10^8	1×10^9
9-12	3×10^8	1×10^9	3×10^9
13-16	1×10^9	3×10^9	1×10^{10}

The vaccine is prepared in 1000 cc of normal saline and administered through a small intraperitoneal catheter which is inserted on the day of treatment and removed after the 1 hour infusion. Catheter placement and diffusion of fluid will be checked by a technitian flow study prior to the vaccine.

8.3 Vaccine side effects. Irradiated tumor cells have been administered as vaccines at intradermal sites in previous studies. At intradermal sites, the local side effects are usually only a transient non-tender bump and even this side effect is largely determined by the adjuvant used rather than the tumor cells. The side effects from intraperitoneal vaccine administration are unknown. The ganciclovir activation of the tumoricidal effect could lead to some local inflammatory response which may be a beneficial antitumor effect. However, whether symptoms will occur is unknown. For this reason the study is designed with an escalating dose plan, the patients will be hospitalized and monitored daily for side effects.

8.4 Ganciclovir is a nucleoside analog. It's an FDA approved drug for the treatment of cytomegalovirus infection. It is excreted by the kidney and a creatinine clearance is a required pretreatment test. Granulocytopenia and thrombocytopenia are common side effects during the 2 to 3 week course for CMV infections. We will only be giving a 7 day course but mild cytopenias can be expected. Pain and phlebitis at the site of administration may occur. Anemia, fever, rash, and abnormal liver enzymes have also been reported.

Reconstitution and administration. It is supplied as a sterile powder and is reconstituted with sterile water. It is not compatible with bacteriostatic water. For IV administration it is prepared in 100 cc of normal saline or

5% Dextrose and water and infused over one hour.

Dosage and adjustments. The standard dose is 5 mg/kg b.i.d. when patients have a creatinine clearance > 80. If the CrCL is 50-79, the dose is 2.5 mg/kg b.i.d.. Daily CBC and platelet counts will be obtained during treatment. The drug should be stopped if the absolute granulocyte count falls below 750 or the platelets are less than 50,000.

8.5 Grading of side effects and toxicity will be determined by the NCI "Common toxicity criteria" (Appendix B).

8.6 To document the patient reported incidence and severity of side effects from treatment, each patient will be asked to complete a self-care diary. Each diary lists 23 possible side effects and includes space for patients to write in additional side effects. Patients also rate the efficacy of self-care activities in alleviating treatment side effects. In addition patients are asked to give an overall rating of how upsetting and disrupting are the side effects. The diary takes approximately 15 minutes to complete. The diary has been shown to have adequate variance and is acceptable to patients (24). It is currently being used with patients receiving traditional chemotherapy as well as inpatients receiving IL-2. The diary will be completed daily for the 7 day hospital stay for each treatment and at 1 week and 2 weeks following discharge.

8.7 Retrovirally transduced PA-1 cells. PA-1 cells will be monitored before vaccine freezing for the presence of replication competent murine retrovirus using the helper rescue assay. In addition, at the time of freezing an aliquot of cells will be taken from the batch and grown in culture for at least two additional months and then assayed for the presence of replication competent virus.

9.0 Measurement of Effects

9.1 Pretreatment immunologic evaluation. Active specific immunotherapy treatments are dependent upon an intact immune system in the host in order to respond to the vaccine. Patients with an impaired immune status due to advanced cancer, nutritional deficiency, or immunosuppressive agents are less likely to respond. The eligibility criteria help to avoid this problem but in addition the status of each patient's immune system will be evaluated as below. Participation is not dependent on the outcome of these tests, but they will be used in analyzing the immunologic and clinical parameters.

Recall skin tests. This tests immune responsiveness to challenge with antigens which virtually all individuals have been exposed to previously: candida albicans, streptokinase/streptodornase, and mumps.

9.2 Immune response directed at autologous tumor cells. The preclinical work suggests that the intraperitoneal vaccine procedure will achieve a systemic immunity. We will evaluate this aspect of the response in two ways.

Autologous tumor cell skin tests. Prior to treatment and subsequent to each vaccine administration, the patients will be challenged with 10^6 radiated autologous tumor cells (if available) given as an intradermal injection. The tissue will have been obtained from the patient's initial surgical resection. We have previously used this skin testing in studies with renal carcinoma. The procedure is described in Appendix A.

Lymphocyte cytotoxic assay. Using the patient's lymphocytes as effector cells and autologous tumor cells as targets, we will determine if a population of cytotoxic lymphocytes is produced by the immunizing procedure. The methods are described in Appendix C.

9.3 Immune responses directed at MHC antigens on the allogeneic ovarian tumor cells of the vaccine. It may be difficult to detect immune responses directed at autologous tumor antigens (sec. 9.2). However, responses to the MHC antigens should definitely occur and be measurable. Although this is a Phase I study and the patient numbers are necessarily small, we will be observing for T cell and B cell responses and comparing low levels of vaccine cell dose with higher levels.

Cytotoxic assays. This will be performed concurrently with the autologous tumor cell assay but the target cell line will be the PA-1 used in the vaccine.

Anti-HLA antibodies. The HLA antigens exposed on the SK OV-3 line will be determined through the tissue typing laboratory. Serum specimens will be serially obtained from each patient to determine an antibody titer directed at the HLA molecule.

9.4 The CA 125 marker level will be determined prior to beginning therapy and prior to each vaccine cycle.

9.5 Imaging studies will be obtained following the second treatment. Another imaging study will be performed following completion of the treatment.

9.6 Laporatomy or laporoscopy. A laporoscopy or laporotomy (presence of adhesions) will be performed within 45 days of beginning this study to document the presence and size of tumor. Following treatment, measurement of the response will be documented by laporoscopy or laporotomy.

10.0 Administrative Aspects

All institutional, National Cancer Institute, State, and Federal regulations regarding informed consent and peer judgment will be fulfilled. See attached consent form.

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Protocol for Gene Therapy of the Respiratory Manifestations of Cystic Fibrosis Using a Replication Deficient, Recombinant Adenovirus to Transfer the Normal Human Cystic Fibrosis Transmembrane Conductance Regulator cDNA to the Airway Epithelium[1]

Document prepared for the NIH Recombinant DNA Advisory Committee

October 7, 1992

Principal Investigator: Ronald G. Crystal, M.D., Chief, Pulmonary Branch, National Heart, Lung, and Blood Institute, Room 6D03, Building 10, Phone 301-496-1597, FAX 301-496-2363

Associate Investigators: Ari Jaffe, M.D., Senior Investigator, Pulmonary Branch; Steve Brody, M.D., Senior Investigator, Pulmonary Branch; Andrea Mastrangeli, M.D., Senior Investigator, Pulmonary Branch; Noel G. McElvaney, M.D., Senior Investigator, Pulmonary Branch; Melissa Rosenfeld, M.D., Senior Investigator, Pulmonary Branch; Chin-Shyan Chu, M.D., Senior Investigator, Pulmonary Branch; Claire Danel, M.D., Special Volunteer, Pulmonary Branch; John Hay, M.D, Visiting Associate, Pulmonary Branch; Tony Eissa, M.D., Senior Staff Fellow, Pulmonary Branch; Thomas M. Horiagon, M.D., Special Volunteer, Pulmonary Branch.

[1]The protocol is also being considered by the Food and Drug Administration as an Investigational New Drug Application from the Pulmonary Branch, National Heart, Lung, and Blood Institute, Ronald G. Crystal, M.D., Principal Investigator.

Precis

This protocol is an initial safety and biologic efficacy study to evaluate the use of the replication deficient recombinant adenovirus AdCFTR to transfer the normal human cystic fibrosis transmembrane conductance regulator (CFTR) cDNA to the respiratory epithelium of adults with cystic fibrosis (CF). If successful, this strategy has the potential to form the basis of therapy to prevent the respiratory manifestations of CF, thus alleviating the major cause of morbidity and mortality of this common hereditary disorder. The overall design is that of a combined ascending dose toxicity study and biologic efficacy study with the patients serving as their own controls. Following a baseline evaluation period and a vehicle control period, AdCFTR will be administered to the left nostril and, one day later, to the left large bronchi. Five groups will be studied (n=2 each group), with each group receiving increasing amounts of AdCFTR. At the completion of the study, the following questions will be answered: (1) Is it safe to administer a vector of the design of AdCFTR to the respiratory epithelium in CF?; (2) Will AdCFTR correct the biologic abnormalities of CF in the respiratory epithelium?; (3) How long does the biologic correction last?; (4) Is the correction sufficient to correct the abnormal electrical potential difference of the airway epithelial sheet?; (5) Is there improvement in clinical parameters relevant to the disease process?; and (6) Does humoral immunity develop against AdCFTR sufficient to prevent repeated administrations in the future?

Outline

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    1.1 General background regarding cystic fibrosis
    1.2 Pulmonary manifestations of cystic fibrosis
    1.3 Current therapy for the respiratory manifestations of cystic fibrosis
    1.4 New therapies being evaluated for the respiratory manifestations of cystic fibrosis
    1.5 Rationale for gene therapy to treat the respiratory manifestations of cystic fibrosis
    1.6 Cystic fibrosis transmembrane conductance regulator (CFTR) gene and mutations
    1.7 CFTR protein and its function
    1.8 Molecular pathogenesis of cystic fibrosis
    1.9 Cell targets for gene transfer in cystic fibrosis

        1.9.1 General airway morphology
        1.9.2 Normal airway epithelium
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        1.9.4 Epithelial lining fluid, pericellular fluid and mucus
        1.9.5 Airway epithelium in CF

    1.10 CFTR gene expression in the airway epithelium
    1.11 Pathogenesis of the respiratory manifestations of cystic fibrosis

        1.11.1 CFTR mutations and respiratory disease
        1.11.2 How abnormal CFTR gene expression causes the characteristic airway disease

    1.12 Rationale for using a replication deficient adenovirus containing the human CFTR cDNA to treat the respiratory manifestations of cystic fibrosis

2. Replication deficient recombinant adenovirus containing the human CFTR cDNA

    2.1 Overview of the adenovirus
    2.2 Structure of the adenovirus

        2.2.1 Structural proteins

    2.3 Structure of the replication deficient, recombinant adenovirus vectors
    2.4 Fabrication and production of AdCFTR
    2.5 Formulation and quality control parameters

3. Experimental evidence supporting the use of a replication deficient recombinant adenovirus containing the human CFTR cDNA to treat the respiratory manifestations of cystic fibrosis

    3.1 <u>In vitro</u> evidence

3.1.1 Expression of CFTR mRNA
3.1.2 Expression of CFTR protein
3.1.3 Functional "complementation" of the cystic fibrosis phenotype
3.1.4 Can the vector survive in the airway epithelial lining fluid of individuals with cystic fibrosis?

3.2 In vivo evidence

3.2.1 Expression in vivo in cotton rats
3.2.2 Expression in vivo in non-human primates

4. Safety considerations

4.1 Will the vector replicate in human epithelial cells?
4.2 Does the vector express viral genes in addition to the CFTR cDNA?
4.3 Is respiratory infection with the vector associated with transfer of the exogenous gene to the germ line?
4.4 Is respiratory administration of the vector associated with damage to the lung?
4.5 Are anti-vector antibodies elicited by respiratory administration of the vector?
4.6 Does repeat administration of the vector pose a risk?
4.7 Is the vector shed following respiratory administration of the vector?
4.8 Are there risks of having some of E3 deleted from the vector?
4.9 Can recombination or complementation of the vector occur in vivo following respiratory administration of the vector, and if so, does this pose a risk to the patient and/or environment?
4.10 Is there a risk of malignancy associated with the use of the vector?
4.11 Does the genome of the vector integrate into the genome of the target cells?
4.12 Does the expression of the CFTR cDNA need to be regulated?

5. Human Protocol

5.1 General design
5.2 Constraints that dictate design of the protocol
5.3 Baseline safety data from individuals with cystic fibrosis relevant to administration of a replication deficient recombinant adenovirus

5.3.1 Anti-vector antibodies in blood and lungs of individuals with cystic fibrosis
5.3.2 Infectious viruses in the respiratory tract of individuals with cystic fibrosis
5.3.3 Baseline inflammation in nasal epithelium, bronchial epithelium and epithelial lining fluid of individuals with cystic fibrosis
5.3.4 DNA containing Ela sequences in nasal epithelial cells, bronchial epithelial cells, lung inflammatory cells, blood lymphocytes and blood neutrophils of individuals with cystic fibrosis

5.4 Patient eligibility and selection

5.4.1 Inclusion criteria
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5.5 Safety and efficacy parameters

5.5.1 Definitions
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5.6 Clinical protocol

5.6.1 Overview
5.6.2 Baseline period
5.6.3 Vehicle control period
5.6.4 AdCFTR experimental treatment period
5.6.5 Management of intercurrent illness
5.6.6 Management of adverse events
5.6.7 Ability of the protocol to answer the questions posed in the introduction to the general design (Section 5.1)

5.8 Additional safety issues

5.8.1 Health care workers
5.8.2 Environment

5.9 Risk-benefit considerations for the patient
5.10 Post-study patient follow-up
5.11 Clinical facilities for the study
5.12 Privacy and confidentiality
5.13 Informed consent
5.14 Reporting of serious adverse affects
5.15 Future directions

6. Protocol in the NIH Recombinant DNA Advisory Committee "Points to Consider" format

7. References

Appendix 1 - Copy of Rosenfeld, M.A. et al. Cell 1992; 68: 143-155

Appendix 2 - Copy of Mastrangeli, A. et al. J. Clin Invest 1992 (in press)

Appendix 3 - Sequence data

Appendix 4 - Approval documents

SECTION 1

INTRODUCTION

1. Introduction

This protocol represents an initial evaluation of the safety and biologic efficacy of the administration of replication deficient recombinant adenovirus containing the human CFTR cDNA to the respiratory epithelium of adults with cystic fibrosis (CF). The protocol is divided into seven sections and 4 appendices, as follows:

Section 1: Provides general background regarding CF, with primary focus on the pulmonary manifestations and current therapy for the disorder, the rationale for gene therapy to treat the respiratory manifestations of CF, the CFTR gene and its mutations, the CFTR protein and its functions, the molecular pathogenesis of CF, the cell targets for gene transfer in CF, CFTR gene expression in the airway epithelium, pathogenesis of the respiratory manifestations of CF, and the rationale for a recombinant adenovirus as the vector of choice.

Section 2: Presents an overview of the adenovirus and details of the fabrication, production, structure, formulation and quality control parameters of the replication deficient recombinant vector containing the human CFTR cDNA.

Section 3: Details the in vitro and in vivo experimental evidence supporting the use of a replication deficient recombinant adenovirus containing the human CFTR cDNA to treat the respiratory manifestations of CF.

Section 4: Summerizes the safety concerns and presents the in vitro and in vivo safety studies in cotton rats and non-human primates regarding the use of a replication deficient recombinant adenovirus containing the human CFTR cDNA.

Section 5: Presents the general design of the human protocol, constraints that dictate design of the protocol, relevant baseline safety data from individuals with CF, criteria for patient eligibility and selection safety parameters to be examined, efficacy parameters to be evaluated, details of the clinical protocol, safety issues for health care workers and the environment, risk-benefit considerations for the patient, plans for doing long term patient follow up, clinical facilities for the study, privacy and confidentiality, informed consent, reporting of serious adverse affects, and future directions.

Section 6: Protocol in the NIH Recombinant Advisory Committee "Points to Consider" format.

Section 7: References

Appendix 1: Copy of Rosenfeld, MA et al Cell 1992; 68:143-155
Appendix 2: Copy of Mastrangeli, A et al J Clin Invest 1992 (in press)
Appendix 3: Sequence data
Appendix 4: Approval documents

1.1 General Background Regarding Cystic Fibrosis

Cystic fibrosis (CF) is a recessive hereditary disorder caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. It is the most common lethal hereditary disorder in the USA (Boat et al., 1989). The disease is confined mostly to the caucasian and hispanic population, although blacks and asians can be affected (Boat et al., 1989; Tsui and Buchwald, 1991). Parents of affected individuals have no clinical manifestations of CF. Males and females are equally at risk. In caucasians, 1 in 20 are heterozygotic carriers and the incidence is 1 in every 3000 live births. In hispanics, the incidence is 1 in 7000 births. Each year, 1,300 people are born in the U.S.A. with CF. There are an estimated 25,000 individuals in the USA with CF, and approximately 50,000 worldwide (Cystic Fibrosis Foundation, Bethesda, Md., personal communication).

The clinical manifestations of CF are primarily in the lung, intestinal tract, pancreas and liver (Boat et al., 1989; Welsh and Fick, 1987). The respiratory manifestations dominate, with thick mucus, chronic airway infections and inflammation beginning in early childhood and leading to progressive loss of lung function. Intestinal problems are most common in infants and include meconium ileus and intussusception. The exocrine glands of the pancreas are destroyed causing pancreatic enzyme deficiency and malnutrition. Diabetes can occur, as can liver failure.

There is no cure for CF. The median survival in the USA is 29 years. Current treatments only ameliorate symptoms. Therapies involve administration of antibiotics, physical chest manipulations for bronchial drainage, physical exercise, oral pancreatic enzymes, vitamins and dietary management. Lung transplantation has been carried out in CF individuals, but the efficacy of this procedure is not proven, and obtaining suitable donor organs is a major problem (Starnes et al., 1992).

The cost of this disease is significant. Beyond the personal tragedies for the affected individuals and their families, there are the associated costs of medical care. The average cost of drugs, diet supplements and medical equipment is $27,500 annually per patient. One to two hospital admissions per year are necessary for 38% of CF individuals. There are significant resources in the USA directed toward developing therapies for CF. In 1991, NIH directed $46.9 million to CF research and the National Cystic Fibrosis Foundation directed $24 million (Cystic Fibrosis Foundation, personal communication). In addition, approximately $30 million was spent by the pharmaceutical industry in trials directed toward therapy for CF (Cystic Fibrosis Foundation, 1991 statistics, personal communication).

1.2 Pulmonary Manifestations of Cystic Fibrosis

Inherent in the concept that the morbidity and mortality associated with CF might be reversed by transferring genes to epithelial cells of the lung is the knowledge that the clinical manifestations of CF are dominated by abnormalities manifest on the epithelial surface of the airways (Elborn and Shale, 1990). In this regard, while CF is also characterized by exocrine

pancreatic insufficiency and high concentrations of sodium chloride in sweat, the pulmonary manifestations are, by far, the most common life threatening aspects of the disease. The earliest observed morphologic lesions are mucous obstruction of small airways and inflammation of the bronchiolar walls (Bedrossian et al., 1976). Bronchoalveolar lavage studies demonstrate neutrophil-dominated inflammation in airway epithelial lining fluid of infants with CF as early as age 1 (Birrer et al., submitted for publication). As the disease progresses, there is inflammation of large and small airways, hypertrophy of submucosal glands, and a general increase in the numbers of secretory cells. There is obstruction of airways with mucous. Chronic infection of the airways develops, with accompanying acute and chronic inflammation. With increasing cycles of mucous obstruction, infection and inflammation, the airways become damaged, culminating in bronchiectasis. Although the disease is primarily based in the airways, the mucus obstruction, inflammation and infection commonly extends to the peribronchiolar alveolar structures, causing fibrosis and alveolar destruction. In the late stages of the disease, the lung is markedly deranged with dilated and sometimes stenosed airways, emphysema, and peribronchiolar and interstitial alveolar inflammation and fibrosis (Bedrossian et al., 1976).

Infection plays a prominent role in the pathogenesis of the derangements of the lung in CF. The infection is primarily endobronchial and is chronic, with acute exacerbations. The most common organisms involved are Hemophilus influenza, Staphylococcus aureus, and Pseudomonas aeruginosa. The fact that the infection is confined to the lung argues strongly that the host defense problems permitting the infection are local rather than systemic (Elborn and Shale, 1990; Kulczycki et al., 1978). The pathobiologic processes permitting chronic infection of the airways are not fully understood, but may involve abnormalities in the volume, physical properties and/or characteristics of mucus in the respiratory tract (Hubbard et al., 1992). In addition, the dysfunctional and/or damaged airway epithelial cells may permit organisms such as P. aeruginosa to adhere to the epithelium, permitting chronic colonization (Woods et al., 1980).

Concomitant with the infection is chronic intense inflammation of the airways that is dominated by neutrophils. Mononuclear phagocytes and lymphocytes play a lesser role. Sputum and lavage fluid analyses of CF patients reveal large concentrations of inflammatory mediators, particularly neutrophil proteases (McElvaney et al., 1991). Bacterial proteases, including Pseudomonas elastase are present, but to a far lesser extent (Doring et al., 1989). Concomitant with the response to the infection, inflammatory cells in the local milieu are chronically releasing large amounts of oxidants (Roum et al., 1990). Together, the mediators released by the inflammatory cells overwhelm the normal anti-inflammatory defense screen of the epithelial surface, and interfere with local host defense processes in the airways (McElvaney et al., 1991; Roum et al., 1990). The inflammatory mediators also exaggerate epithelial cell secretion (Sommerhoff et al., 1990), thus perpetuating the increased mucus production that characterizes the disease. Consequent to this chronic, overwhelming inflammation, there is progressive damage to the epithelium culminating in the bronchiectasis and other permanent derangements of the lung that characterize CF.

The clinical manifestations of CF reflect the progressive derangements to the airways (Boat et al., 1989). Early in the disease there is cough, together with respiratory tract infection. The sputum becomes thick and purulent. There is a long period of chronic bronchitis with acute exacerbations. Eventually, the permanent derangements of the respiratory tract cause symptoms of shortness of breath. The cycles of chronic and acute infection eventually culminate in limitation of activity, weight loss, and end-stage lung disease with hypoxemia, pulmonary hypertension, cor pulmonale and death (Boat et al., 1989; Shwachman and Kulczycki, 1958). Even with aggressive treatment to control infection, clear the airways of secretions, and improve nutrition, the 1991 survival data for CF in the USA demonstrates that only 50% survive beyond the age of 29 (Patient registry data, Cystic Fibrosis Foundation).

1.3 Current Therapy for the Respiratory Manifestations of Cystic Fibrosis

Current therapy for the lung disease in cystic fibrosis requires a multidisciplinary approach of outpatient and inpatient care. Outpatient therapy includes frequent postural drainage and chest percussion, administration of antibiotics, and bronchodilators. While these therapies are used by most clinicians caring for patients with CF, the therapy often varies in different centers. For example, while most clinicians agree regarding the benefits of postural drainage and chest percussion, some advocate it for all patients while others reserve it for those with copious sputum production. Likewise, some centers advocate continuous antibiotic therapy while others reserve antibiotics for documented infections. Bronchodilator therapy may give symptomatic relief to many individuals with CF but the response is not universal. Use of other therapeutic strategies such as mucolytics, expectorants and chronic corticosteroids or other anti-inflammatory agents remain unproven. Early vaccination for measles and pertussis is important and yearly influenza vaccination is advocated.

Inpatient therapy is necessary when there are exacerbations of pulmonary infections or complications of CF lung disease such as hemoptysis, pneumothorax, respiratory failure, pulmonary hypertension, and cor pulmonale. For acute infections requiring hospitalization, empiric antibiotic therapy is necessary at first but is then based on culture and sensitivity results from respiratory secretions. Patients with acute infections are also given increased postural drainage and chest percussion, bronchodilator therapy, and occasionally corticosteroids. The treatment of the lung complications of cystic fibrosis does not differ from the treatment of other chronic lung disease states.

1.4 New Therapies Being Evaluated for the Respiratory Manifestations of Cystic Fibrosis

New strategies to treat cystic fibrosis include aerosol therapy with recombinant human DNase (to cleave DNA in purulent respiratory secretory, thus helping to clear the airways of the thick, infected mucus), aerosolized plasma α1-antitrypsin or recombinant human secretory leukoprotease inhibitor (to inhibit neutrophil elastase on the respiratory epithelial surface,

thus protecting the epithelium from the damaging effects of neutrophil elastase), aerosolized reduced glutathione (to augment the antioxidant protective screen of the airway to protect the epithelium from the inflammatory cell derived oxidant burden) and aerosolized amiloride (to reestablish the ionic milieu of the epithelial surface) (Hubbard et al.,1992; McElvaney et al.,1991; McElvaney et al., 1992; Knowles et al., 1990; Roum, J. and Crystal, R.G. unpublished observations). Although there are encouraging results from all of these agents, all have been evaluated only in phase I or II studies, and none attack the primary abnormality in the disease. Lung transplantation, including heart-lung, double lung and single lung procedures has been carried out in 312 individuals with CF (worldwide through 1991). The three year survival rate in this cohort is 52%. The average cost of these procedures is $150,000-200,000, with an estimated cost of $28,000/year for follow up treatment. Finding suitable donors is a major problem, with typical waiting periods of 9 to 12 months.

1.5 Rationale for Gene Therapy to Treat the Respiratory Manifestations of Cystic Fibrosis

The identification of the CFTR gene in 1989 (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989) opened the door to strategizing therapies for CF in which the normal gene would be transferred to somatic cells of individuals with CF, thus reversing the biologic abnormalities consequent to mutations of the two parental CFTR genes. Because the respiratory manifestations of CF dominate the clinical picture, it is the most rational target for gene therapy for this disorder. The concept that the respiratory manifestations of CF are a good candidate for gene therapy is based on several facts. First, the respiratory disease is confined to the epithelium of the airways. In vitro studies have demonstrated that the CFTR protein is a Cl^- channel that modulates the secretion of Cl^- in response to elevations of intracellular cAMP (Anderson et al., 1991a; Anderson et al., 1991b; Bear et al., 1992; Drumm et al., 1990; Rich et al., 1990). Mutations of the CFTR gene render epithelial cells unable to modulate Cl^- permeability through the cAMP pathway (Frizzell et al., 1986; Hwang et al., 1989; Li et al., 1988; Li et al., 1989). It is this biologic abnormality that is believed to cause the respiratory manifestations of the disease (Collins, 1992; Welsh et al., 1992; Welsh and Fick, 1987). Second, in vitro studies have shown that transfer of the normal CFTR cDNA to epithelial cell lines derived from individuals with CF can override this abnormality and permit the cells to secrete Cl^- in response to increased intracellular cAMP (Drumm et al., 1990; Rich et al., 1990). Finally, the lethal consequences of mutations of the gene occur almost exclusively in the lung (Welsh and Fick, 1987). Together, these concepts suggest the feasibility of somatic gene therapy for CF, i.e., it should be possible to correct the pulmonary manifestations caused by mutations of the CFTR gene by directly transferring a normal CFTR cDNA to airway epithelial cells.

The architecture of the airways demands that if gene therapy for the respiratory manifestations of CF is to be successful, the transfer of the normal CFTR cDNA to the airway epithelium will have to be carried out in vivo via the air side of the epithelium. On clinical and technical grounds it is not

possible to use ex vivo strategies to remove the epithelium, insert the normal cDNA and replace the existing epithelium. The adult human airways have a surface area of 1-2 m^2. There are at least 6 major epithelial cell types, with the majority of the cells terminally differentiated. Human airway epithelial cells can be cultured, but the methods are primitive, the differentiated state of the cells is not necessarily the same as that in vivo, the growth factors are not known, the normal cell ontogeny is not clearly defined, nor is the airway epithelial stem cell population (Rennard et al., 1991). Most importantly, the dichotomous branching nature of the airways precludes any strategies to remove the epithelium and/or introduce corrected autologous airway epithelial cells. Together, these facts argue strongly for an in vivo approach to gene therapy. The anatomy dictates that this is feasible only through the air side of the epithelium. Unlike the lower respiratory tract which gets its blood supply from the pulmonary capillaries, the airways are supplied by the bronchial circulation, an arterial system comprised of multiple branches derived from the aorta (Deffebach and Widdicombe, 1991). Although it is feasible to place catheters into the bronchial circulation, their multiplicity and variability make this approach very cumbersome. Further, even if the transfer vector could be delivered to the airways via the bronchial circulation, the cDNA would have to cross the endothelium, the endothelial basement membrane, the interstitial space, and the epithelial basement membrane before entering the basolateral surface of the epithelium, a very unlikely possibility.

1.6 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Gene and Mutations

The gene responsible for CF, the CFTR gene, is a 27 exon gene spanning over 250 kb on the long arm of human chromosome 7 at q31-q32 (Riordan et al., 1989; Rommens et al., 1989; Zielenski et al., 1991). The encoded mRNA is about 6500 nucleotides in length. The sizes of the 27 exons vary widely, with exon 14b the smallest (38 bp) and exon 13 the largest (724 bp) (Figure 1.6-A). Sequence analysis of approximately 10% of the entire CFTR gene has shown that all intron/exon junction sequences obey the GT-AG consensus rule. The gene includes a number of repetitive elements in introns, including 5 Alu repeats and 1 Kpn repeat and several simple repeats (microsatellites), such as $(GT)_{17}$, $(GT)_{12}$, $(GATT)_7$, and $(TA)_{14}$.

The structure of the putative CFTR gene product is a 1480 residue glycoprotein. There is (N- to C-terminal) a membrane-spanning domain with six membrane-spanning segments, a nucleotide (ATP)-binding fold (NBF), a large polar R (regulatory) domain which contains multiple potential phosphorylation sites, a second similar membrane-spanning domain and a second NBF (Figures 1.6-A, 1.6-B) (Riordan et al., 1989).

Approximately 220 sequence variations of the CFTR gene have been identified, of which about 170 are associated with the clinical manifestations of CF. The mutations include missense mutations, nonsense mutations, frameshift mutations, splicing mutations, and small deletions and insertions (Collins, 1992). Most of these mutations are scattered throughout the coding region of the gene. Many different mutations have been found at the

Figure 1.6-A. The cystic fibrosis transmembrane conductance regulator (CFTR) gene, mRNA and predicted protein product. The CFTR gene is comprised of 27 exons (denoted 1-5, 6a, 6b, 7-13, 14a, 14b, 15-16, 17a, 17b, 18-24) spread out over 250 kb of chromosome 7. The mRNA is 6.5 kb, including 4.5 kb of coding exons and a 2 kb untranslated 3′ tail (not shown). The predicted CFTR protein has 5 major domains as indicated (see Figure 1.6-B for details concerning the structure and function of the CFTR protein). Also indicated is the location in exon 10 of the common ΔF508 mutation (see text, section 1.6).

Figure 1.6-A

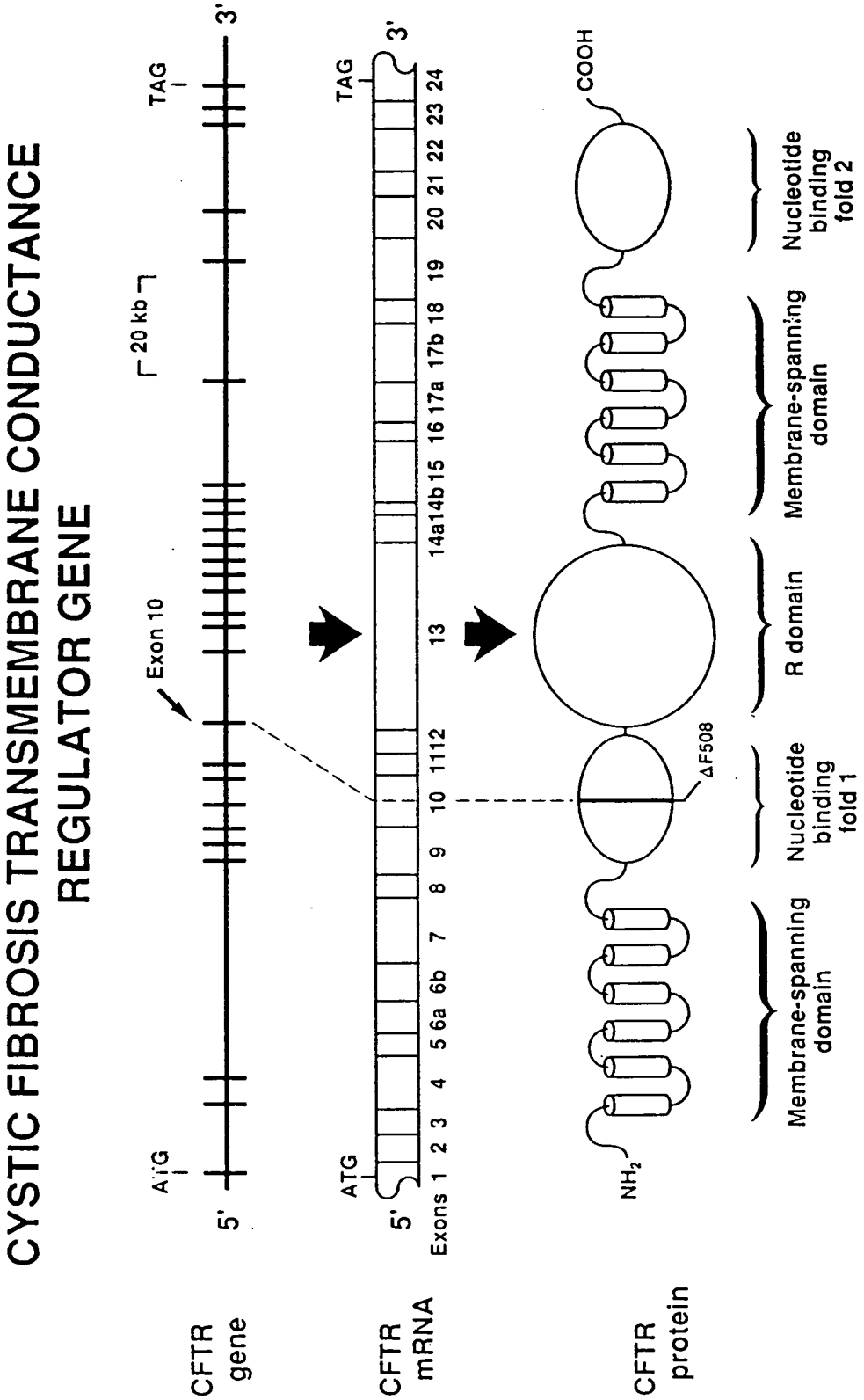
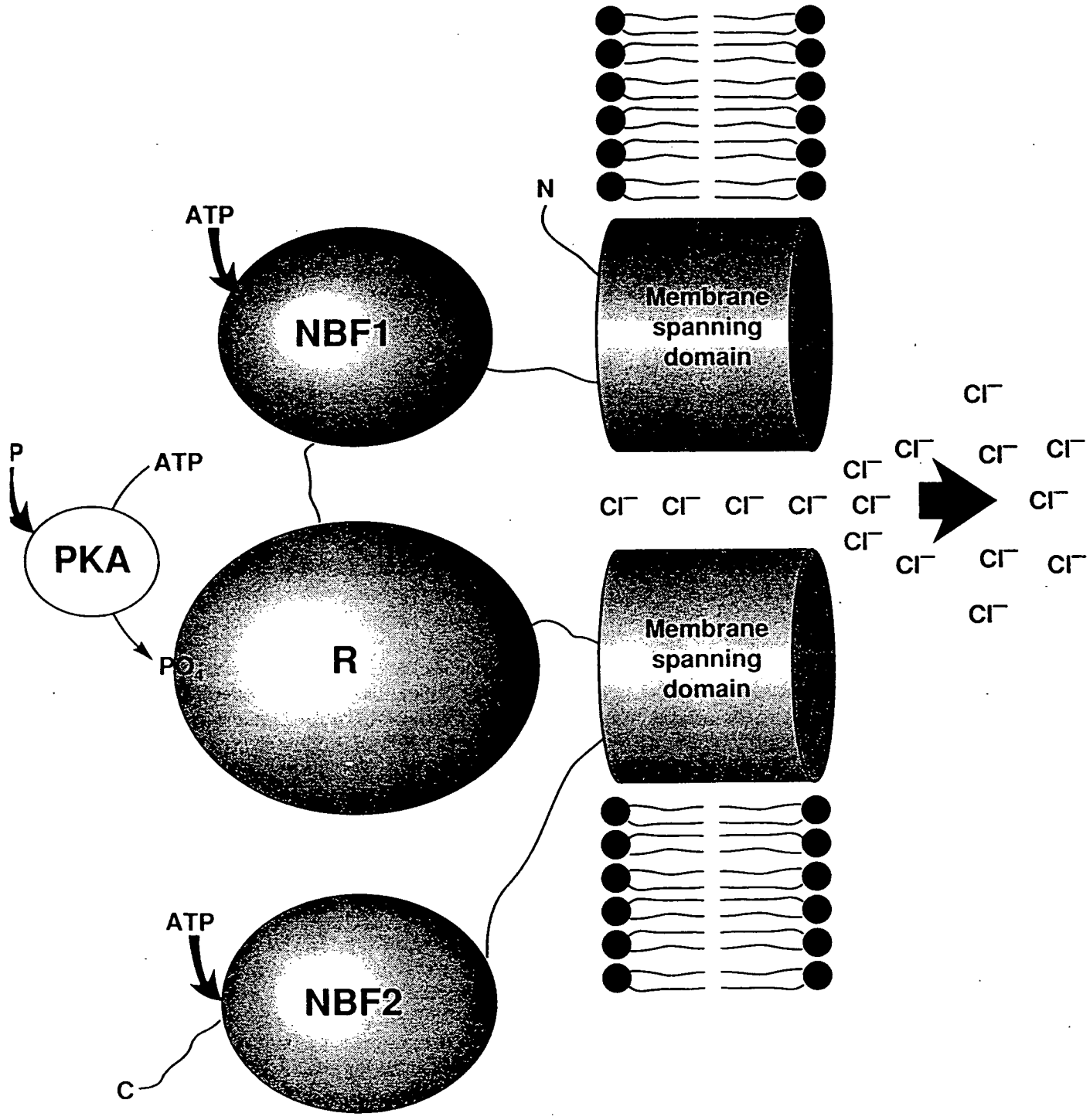

CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR GENE
CFTR gene
CFTR mRNA
CFTR protein
5'
3'
ATG
TAG
20 kb
Exon 10
Exons 1 2 3 4 5 6a 6b 7 8 9 10 11 12 13 14a 14b 15 16 17a 17b 18 19 20 21 22 23 24
NH2
COOH
ΔF508
Membrane-spanning domain
Nucleotide binding fold 1
R domain
Membrane-spanning domain
Nucleotide binding fold 2

Figure 1.6-B. Structure and function of the cystic fibrosis transmembrane conductance regulator (CFTR) protein. The predicted structure is that of a membrane bound single chain polypeptide with (N- to C-terminal): an N-terminal intracytoplasmic portion, a membrane spanning domain (comprised of 6 individual membrane spanning segments), nucleotide binding fold 1 (NBF1), a regulatory (R) region, a second membrane spanning domain (similar to the first), nucleotide binding fold 2 (NBF2), and a C-terminal intracytoplasmic tail. Data from a variety of sources have demonstrated the CFTR protein functions as a Cl^- channel responsive to increased levels of cAMP. Activation of the channel requires binding of ATP to NBF1 and NBF2, and cAMP activation of protein kinase A (PKA) to phosphorylate residues in the R domain.

Figure 1.6-B


N
ATP
NBF1
Membrane spanning domain
P
ATP
PKA
R
PO_4
Membrane spanning domain
ATP
NBF2
C
Cl⁻

highly conserved region of the first NBF (Collins, 1992), suggesting that the first NBF is important to CFTR function. The most common mutation is ΔF508, a deletion of 3 nucleotides resulting in loss of phenylalanine at residue 508, a region coded by sequences in exon 10 in NBF1. ΔF508 accounts for 60 to 70% of the CF mutant alleles (Kerem et al., 1990). G551D, S549I, A455E, and G542X account for 10-20% of the non-delta F508 mutations (Kerem et al., 1990). Most other mutant alleles are rare, with some represented by only a single example.

Studies of correlations between specific mutations and severity of disease have shown some variation in severity even in individuals with the same genotype. For example, the severity of the respiratory disease in ΔF508 homozygotes can be variable although most of these individuals have relatively severe pancreatic insufficiency (Kerem et al., 1990). Frameshift, nonsense, and splicing mutations have been found in compound heterozygotes [e.g., G542X/S1255X or R553X/W1316X]. Interestingly, despite the fact that these individuals cannot produce CFTR, they only have mild pulmonary disease, suggesting that the absence of CFTR is not incompatible with life (Cutting et al., 1990; Hamosh et al., 1991).

1.7 CFTR Protein and Its Function

The CFTR protein predicted from the CFTR gene is a 1480 single chain glycosylated polypeptide (Figure 1.6-A, 1.6-B). Although the R-domain is unique to CFTR, the two membrane spanning domains and the two nucleotide-binding folds that bind and cleave ATP are also features of proteins in the traffic ATPase/ABC transporter super family that includes prokaryotic periplasmic permeases, the STE6 yeast mating factor, and the P-glycoprotein multidrug resistance protein (Hyde et al., 1990). There are two predicted carbohydrate side chains, both in an external loop of the C-terminal membrane spanning domain.

Localization of the CFTR protein has been difficult because many of the antibodies generated against predicted peptides cross react with unidentified cellular components. It is generally accepted, however, that the protein is present in epithelial tissues and that it is present in cell membranes (Cheng et al., 1990; Crawford et al. 1991; Denning et al., 1992; Marino et al., 1991; Sarkadi et al., 1992). CFTR has been localized to the apical membranes of pancreatic ducts, intestinal epithelia, sweat ducts, and airway epithelia. There is indirect functional data suggesting CFTR may also be localized to organelle membranes, including the Golgi apparatus (Barasch et al., 1991).

There is convincing evidence that CFTR can function as a cAMP regulatable Cl^- channel (Bear et al., 1992). There is also evidence that CFTR may have other functions, such as recycling of vesicles (Bradbury et al., 1992). The regulation of CFTR as a Cl^- channel is not completely defined, but it requires phosphorylation of the R-domain mediated by cAMP activation of protein kinase A (Cheng et al., 1991; Picciotto et al., 1992).

1.8 Molecular Pathogenesis of Cystic Fibrosis

In normal cells expressing the CFTR gene, the CFTR protein is produced as an unglycosylated polypeptide that subsequently undergoes core glycosylation in the rough endoplasmic reticulum, translocation to the Golgi, modification of the carbohydrate side chains to their mature form, and final transfer to the apical membrane (Cheng et al., 1990). In the common ΔF508 mutation, there is a deletion of Phe^{508} in the first NBF. For unknown reasons, glycosylation of this protein is incomplete, and normal translocation to the apical membrane does not occur (Cheng et al., 1990). Cells homozygous for the ΔF508 mutation do not store the mutated form of CFTR, and it is likely degraded (Cheng et al., 1990). Other CFTR mutations such as ΔI507 and S549I have a similar pattern of incomplete glycosylation, but other mutations of CFTR code for a CFTR protein glycosylated in a normal fashion (Cheng et al., 1990; Gregory et al., 1991).

For the common ΔF508 mutation, the central abnormality appears to be the lack of translocation of the mutant protein, since delivery of the ΔF508 protein to the surface of the cell conveys to the cell the ability to secrete Cl^- in response to cAMP, although the ΔF508 Cl^- channels have somewhat reduced activity compared to normal (Dalemens et al., 1991; Drumm et al., 1991).

There is evolving evidence that CFTR may also function within intracytoplasmic organelles (Barasch et al., 1991; Van Dyke et al., 1992). One consequence of a lack of CFTR function at these sites may be a higher pH within the organelle, causing dysfunction of intraorganelle components such as enzymes that modify carbohydrate side chains of proteins such as mucins (Barasch et al., 1991).

1.9 Cell Targets for Gene Transfer in CF

The central purpose of this protocol is to transfer the normal CFTR cDNA in vivo to the airway epithelial cells of individuals with CF. Before considering how the gene will be transferred, it is important to consider which cells should be the targets for the gene transfer. While all evidence suggests the abnormal CFTR gene is expressed in airway epithelial cells, it is not known specifically which airway epithelial cells play a critical role in the pathogenesis of the disease i.e., it may be a subset of cells or it may be all airway epithelial cells. Since the ultimate goal is gene therapy to compensate for the underlying genetic abnormality of CF, it is important to define the current concepts of the biology of airway epithelial cells. All evidence suggests that the alveolar epithelium is not a primary site of the clinical manifestations of mutations of the CFTR gene, and thus the following discussion focuses only on airway epithelium.

1.9.1 General Airway Morphology

The airways of the normal human lung begin at the trachea and end in the alveoli, the site of gas exchange (see Wiebel, 1991 for general review). The total surface area in the human lung is approximately 140 m^2. Most of this surface is in the alveoli, with the airway surface representing approximately 1-2% of the total (E. Weibel, personal communication). The

tracheobronchial tree is comprised of an average of 23 generations of dichotomous branching tubes, ending in the alveoli (see Weibel, 1991 for review) (Figures 1.9.1-A, 1.9.1-B). The region from the trachea to about the 16th generation is referred to as the "conducting zone", structures that serve to conduct air from the external environment to the gas exchanging regions. The airways in this region are referred to as "bronchi". By the 16th generation, there are approximately 66,000 bronchi. From the 17th through the 20th generations are the "bronchioles", the smallest airways. From the 17th generation ending in the alveoli is referred to as the "transition" and "respiratory" zones, the latter being the actual site of gas exchange. By the 20th generation, there are 10^6 airways and by the 23rd generation, $3x10^8$ alveoli.

The walls of bronchi consist of three concentric tubes (Figures 1.9.1-C, 1.9.1-D): (1) a mucosa comprised of an epithelial cell layer resting on a basement membrane; (2) a sleeve of smooth muscle cells; and (3) an outer tube of connective tissue matrix that includes islands of cartilage and submucosal glands that open onto the epithelial surface. The epithelial layer is columnar and pseudostratified; all components abut the underlying basement membrane. On the average, the bronchial epithelium is about 20 μm thick. The entire mass of a large bronchus is about 60 μm in thickness. The submucosal glands are in highest concentration in the trachea, where there is approximately one gland per mm^2 of epithelial surface area. The number of glands becomes sparser toward the periphery, and are absent in the bronchioles. In cross section, the submucosal glands are about 200 μm in width. From the opening of a submucosal gland on the epithelial surface, the gland continues into a 250-500 μm collecting duct lined by columnar epithelium (Figure 1.9.1-C). Up to 13 tubules arise from the collecting duct; these tubules branch extensively and are closely intertwined. The entire submucosal gland is internal to a basement membrane that is continuous with the basement membrane of the airway epithelial cell layer.

The walls of the bronchioles are composed of the same three concentric tubes as the bronchi, but with some differences (Figure 1.9.1-D): (1) a mucosa composed of an epithelial cell layer resting on a basement membrane; (2) a thin sleeve of smooth muscle cells; and (3) a delicate tube of connective tissue matrix, but without cartilage or submucosal glands. Like the bronchial epithelium, the epithelium of the bronchioles are 1 cell thick. The cells are columnar, but less so than in the bronchi.

The blood supply to the airways is primarily from the bronchial arteries, derived from the aorta and intercostal arteries. The bronchioles also derive some blood from the pulmonary artery. Except for a few bronchial veins in the hilar region, the bronchial circulation drains into the pulmonary veins. Together, the anatomic features of the blood supply to the airways make it very difficult to strategize a means to target vectors for gene transfer directly to the bronchial and bronchiolar epithelium by means of the circulation.

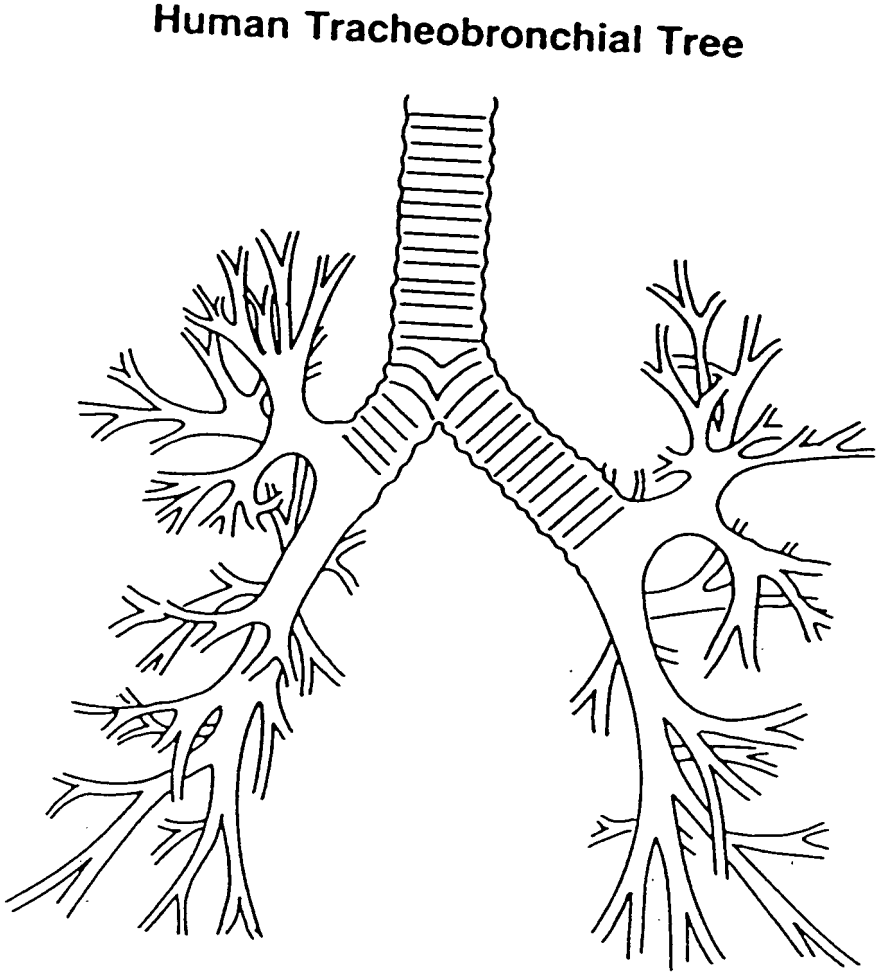


Figure 1.9.1-A. Schematic of the normal human tracheobronchial tree. Shown are the airways branching in a dichotomous fashion from the trachea downward.

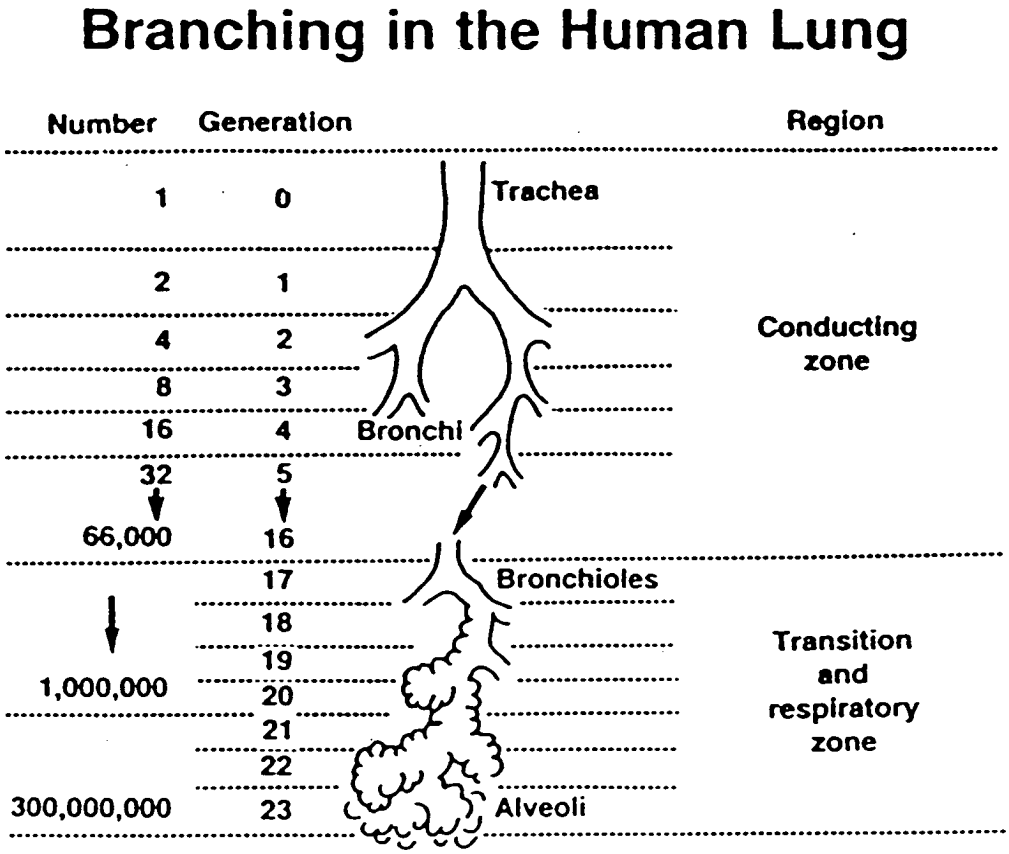


Figure 1.9.1-B. Branching in the normal human lung. The conducting zone represents the region from the trachea through generation 16. The bronchi start at generation 1 and go through generation 16 while the bronchioles start at generation 17 and go through generation 20. By the end of the conduction zone there are approximately 1,000,000 bronchioles.

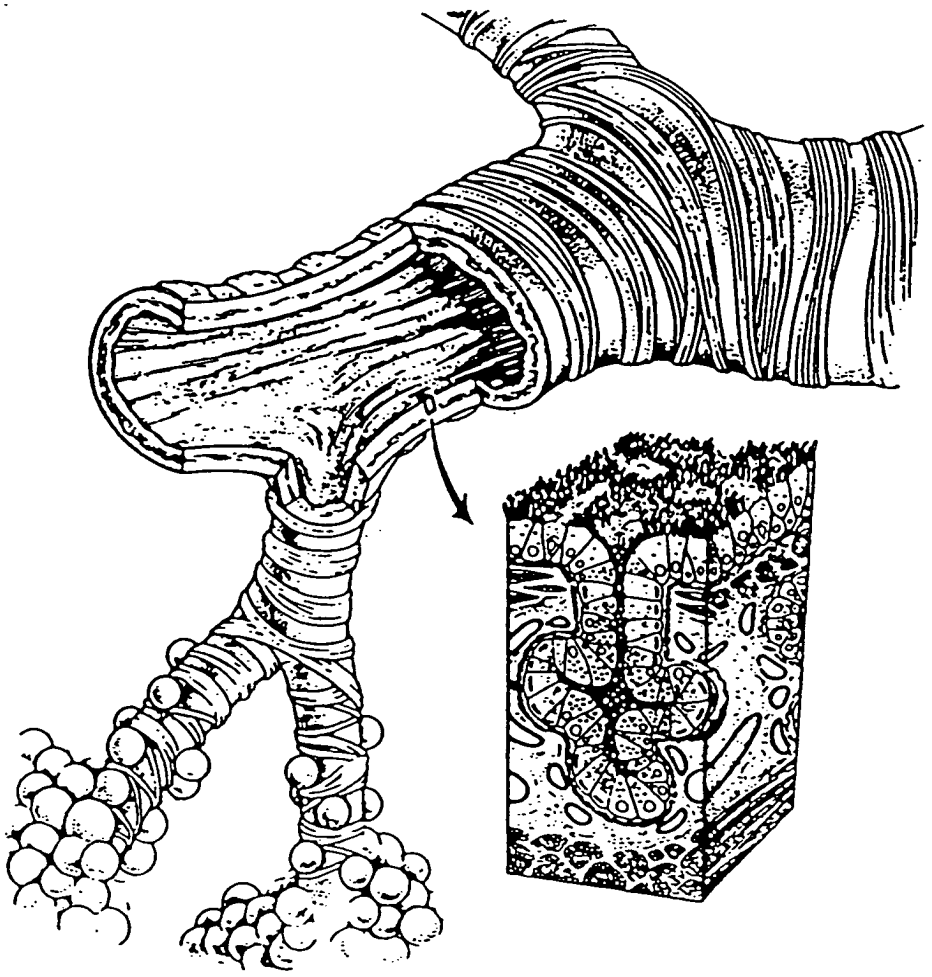


Figure 1.9.1-C. Schematic of the anatomy of the normal bronchial tree with a cut-away view of the epithelium and submucosal bronchial glands. Further, details for the epithelium of the bronchi, submucosal glands, and bronchioles are described in Figures 1.9.1-D, 1.9.2-A, 1.9.2-B. After generation 20 the respiratory bronchioles open into the alveoli, the air exchanging region of the lung. For illustration purposes, a number of branches between the large airways and bronchioles are not shown.

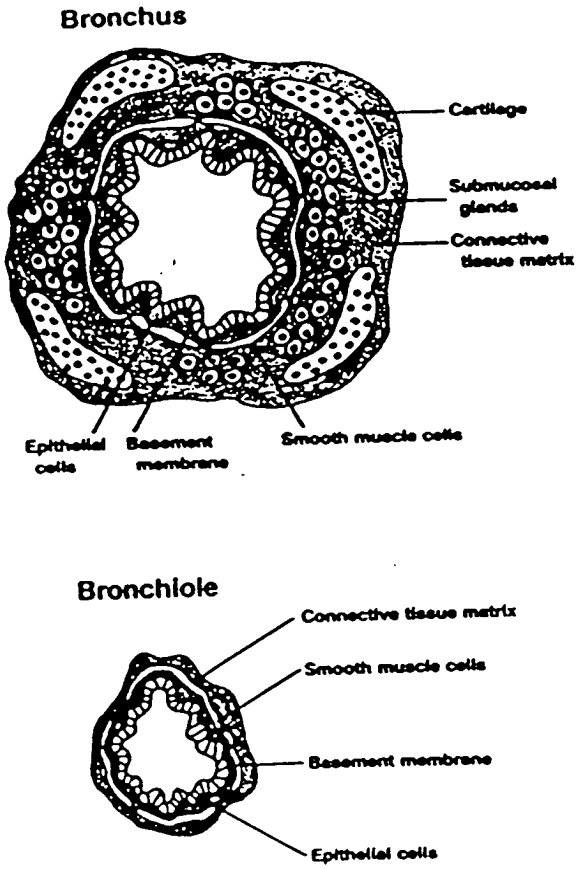


Figure 1.9.1-D. Schematic demonstrating the architecture of the walls of bronchi and bronchioles. Top. Cross-sectional view of a bronchus. It is comprised of three concentric tubes including an inner epithelial layer resting on a basement membrane, a middle sheath of smooth muscle cells and outer layer of connective tissue, islands of cartilage, and submucosal glands that are contiguous with the airway epithelium. Bottom. Cross-sectional view of the walls of a bronchiole. It is also composed of three concentric tubes but the outer layer contains no cartilage and no submucosal glands.

1.9.2 Normal Airway Epithelium

Together, the epithelium of the bronchi, submucosal glands, and bronchioles is comprised of 6 major cell types: ciliated cells, goblet cells, serous cells, undifferentiated columnar cells, basal cells, and Clara cells (Figures 1.9.2-A, 1.9.2-B; see Breeze and Wheeldon, 1977; Basbaum and Finbeiner, 1989; Massaro, 1989 for general reviews). Although some investigators describe the goblet and serous cells in the submucosal glands separately from their counterparts in the epithelial cell layer on the air surface, as there is no evidence they are clearly different or play a different role in CF, they will be considered together. Also, airway epithelium includes a number of minor cell types (minor in number, not necessarily in relevance to airway function) such as "K cells" (neuroepithelial cells), brush cells, and mast cells; however, as there is no evidence these cells play a primary role in the pathogenesis of CF, they will be ignored for this review.

Ciliated cells - these columnar cells are found throughout the airways. In the bronchial epithelium there are approximately 5 times more ciliated cells than any other cell type. Ciliated cells form the lining of the opening of the collecting ducts of the submucosal glands. In the bronchioles the ciliated cells also dominate in numbers, but less so than in the upper airways. The cytoplasm of ciliated cells is relatively sparse, but rough endoplasmic reticulum (RER) and Golgi apparatus are present. On their apical surface, each cell contains approximately 250 cilia, each 6 μm x 0.3 μm. The cilia beat in a coordinated fashion toward the pharynx at about 12-20 Hz.

Goblet cells - also referred to as "mucus cells", these columnar cells are called "goblet" cells because they are shaped like wine glass in longitudinal section. Goblet cells are present in the bronchial epithelium and the submucosal glands. In normal bronchi, there are approximately 6.8×10^3 goblet cells/mm^2. Toward the peripheral airways, their numbers progressively decrease, and they are absent in the bronchioles. Goblet cells are characterized by a cytoplasm dense in mucus granules and extensive RER and Golgi. The mucus granules coalesce toward the apical surface and are secreted through pits or pores on the surface. When discharged from the surface, the granules often retain an intact limiting membrane, although they are also discharged as confluent clumps.

Serous cells - these columnar cells are common in the submucosal glands, but are also present in the surface epithelium of the bronchi, although to a lesser extent than goblet cells. The cytoplasm contains a variable number of secretory granules. The serous cells have a small number of surface microvilli. The contents of the secretory granules are discharged as droplets that individually fuse with the surface.

Undifferentiated columnar cells - these are columnar cells of similar dimensions to ciliated cells. The cytoplasm is relatively sparse and, as the name suggests, the cell has no special features. These cells are found in bronchi, glands, and bronchioles. In bronchi, these cells represent approximately 12% of the airway epithelial cells (Breeze and Wheeldon,

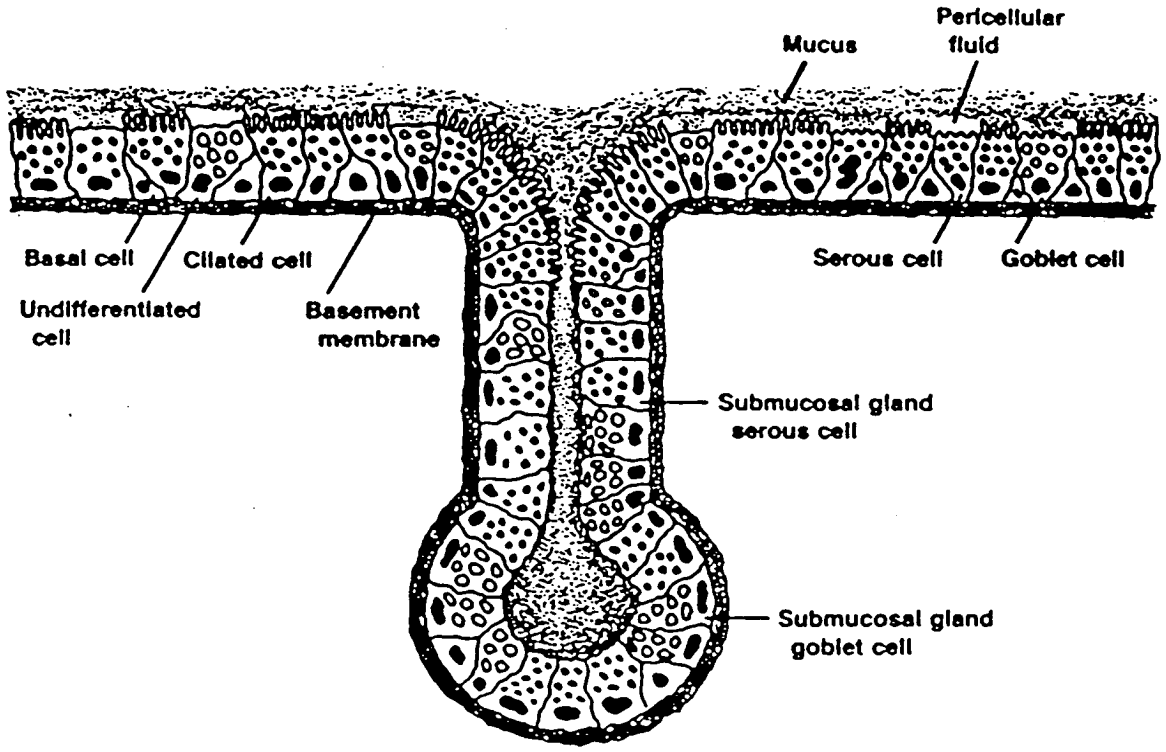


Figure 1.9.2-A. Major cell types of the bronchial wall. The epithelium is pseudostratified, with all cells resting on the basement membrane. The major cell types include ciliated cells, goblet cells, serous cells, undifferentiated columnar cells, and basal cells. The branched tubular structures comprising the submucosal glands are lined with serous and goblet cells. The basement of the airway epithelium glands is contiguous with that of the airway epithelium. Mucus is produced by the goblet and serous cells on the airway epithelial surface and in the mucus glands. Typically, the lumen of the submucosal glands are filled with mucus. Above the airway epithelium, the airway epithelial lining layer consists of a lower phase of pericellular fluid and upper phase of mucus. In the normal lung, the mucus layer is discontinuous, forming "islands" resting on the airway surface.

Bronchiolar epithelium

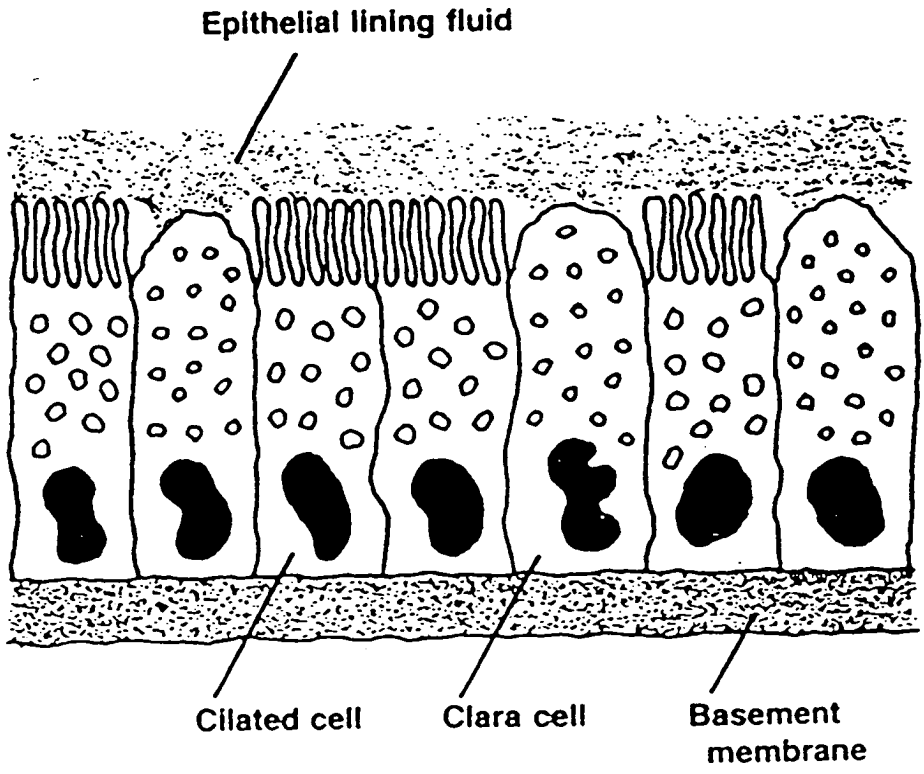


Figure 1.9.2-B. Major cell types of the walls of the bronchioles. The epithelium is columnar and rests on a basement membrane. There are two major cell types; ciliated cells and Clara cells. There are no submucosal glands. The airway epithelial lining fluid is thinner than observed in the larger airways and a distinct mucus layer is not apparent.

1977). They likely differentiate into other cell types, and could function as a "stem" cell for other airway columnar cells (see below).

Basal cells - these are ovid or pyramidal shaped cells scattered along the basement membrane throughout the airways, although more frequently in large bronchi. In bronchioles, basal cells represent <20% of the epithelial cells (Danel et al., 1992; Jeffrey and Reid, 1975). The nucleus fills most of the cell; there are no secretory granules, but some RER and Golgi. Basal cells have a broad based surface on the basement membrane to which they are tightly attached by hemidesmosomes (Kawanami et al., 1979).

Clara cells - this is a columnar cell with an apical surface bulging into the airway lumen. Clara cells are confined to the bronchioles, and are sometimes referred to as "nonciliated bronchiolar epithelial cells" (Massaro, 1989). The Clara granules do not coalesce. These cells are believed to contribute significantly to bronchiolar secretions.

1.9.3 Normal Epithelial Cytokinetics

In the normal lung, the airway epithelium constantly turns over, but at a slow rate (Breeze and Wheeldon, 1977; Evans and Shami, 1989). Most data is from experimental animals and it varies by species (Frasca et al., 1968). Mitoses are rarely seen (Breeze and Wheeldon, 1977). At any given moment, approximately 1 of 200 cells are synthesizing DNA (Greisin, 1977). Estimates of complete cell turnover (100% of the epithelial cells replaced) in the bronchi of experimental animals vary from 7 to 21 days up to 82 to 131 days (Blenkinsopp, 1967; Shorter et al., 1964; Spencer and Shorter, 1962). Cell turnover is more rapid in larger airways with estimates of up to 3-fold in large compared to small bronchi (Bolduc and Reid, 1967). There is no quantitative data relating to epithelial cell turnover in the human lung.

The classic concept of airway epithelial cell ontogeny held that the basal cell was the stem cell for all of the major airway epithelial cells (Evans and Shami, 1989). This view has been modified somewhat to the concept that basal cells regenerate themselves and likely some columnar cells, but that non-ciliated columnar cells can also regenerate columnar cells. In this regard, while ciliated cells are considered to be terminally differentiated, mucus, serous, undifferentiated, and Clara are all capable of replication. In the bronchioles, Clara cells likely serve as the progenitor for other Clara cells and ciliated cells. In the submucosal glands, the progenitor cells are not clear. It has been hypothesized that the undifferentiated columnar cells are the progenitor for Goblet and serous cells but this is not proven. In response to injury, all of the non-ciliated columnar cells undergo division, on the average they spend 8-12 hours in S phase, 2-3.5 hours in G2 and 0.6 hour in M (Boren and Paradise, 1978).

1.9.4 Epithelial Lining Fluid, Pericellular Fluid and Mucus

The surface of the airway epithelium is covered with epithelial lining fluid (ELF) that is 8 to 16 μm thick in the large airways. In the bronchi, electron microscopic observations demonstrate the airway ELF consists of

two layers: (1) Pericellular fluid (PCF) - the layer next to the cells, sometimes referred to as the "sol" phase; and (2) mucus - the upper layer, referred to as the "gel" phase (Figures 1.9.2-A, 1.9.2-B).

(1) PCF is approximately 6 μm in depth and approximately the height of cilia (Massaro, 1989). It is believed that PCF allows cilia to move freely, with the tips of cilia imbedded into the upper mucus layer, permitting the cilia to propel the mucus toward the pharynx (see below). The composition of PCF is not fully defined, but includes proteins as well as small molecules. These components are derived from plasma, produced locally, and include alveolar epi thelial fluid molecules that have moved to the airways.

(2) Mucus - in normal airways, the upper mucus layer is a discontinuous blanket forming mucus islands floating on the PCF (see Basbaum and Finbeiner, 1989; Kaliner et al., 1988; Lopez-Vidriero et al., 1977 for reviews). Mucus is a complex viscoelastic material that contains mucins, proteins, lipids, and proteoglycans. It traps inhaled particulates and, together with the cilia that propel the mucus upward, serves a major defense mechanism to remove inhaled material and endogenous debris from the lung. In the phase of their forward beat, the cilia grasp the mucus blanket with fine claws at their tips.

It is likely that all columnar airway epithelial cells contribute to airway ELF. The goblet and serous cells are the major contributors to the mucus layer (at least the mucin part) (Basbaum and Finbeiner, 1989; Kaliner et al., 1988; Lopez-Vidriero et al., 1977). The physiologic stimuli that regulate mucus production of the bronchial epithelium are largely unknown. In the submucosal glands, macromolecular secretion can be modified by variety of neural influences as well as by a variety of mediators (Kaliner et al., 1988). Glandular serous cells and goblet cells appear to be under somewhat different control. In addition to mucus and PCF, airway ELF likely also contains components of alveolar ELF that has been transported upward.

1.9.5 Airway Epithelium in CF

As the disease progresses, the mucus obstruction, infection and neutrophil-dominated inflammation takes its toll on the normal epithelial architecture (Figure 1.9.5-A). There is no quantitative data available from biopsy samples of individuals with CF. To quantitate these changes in the surface epithelium of the large airways, a fiberoptic bronchoscope was used to collect cytologic brush samples of the bronchial epithelium of individuals with CF (18 years or older, all with moderate lung disease) compared to normal, non-smokers. For comparison, the nasal epithelium was evaluated (Table 1.9.5-B). The data demonstrates that while the nasal epithelium is similar in normals and those with CF, there are significant changes in the proportions of epithelial cell types in the large bronchi, with CF individuals having fewer ciliated cells, but increased proportions of basal, secretory and undifferentiated columnar cells.

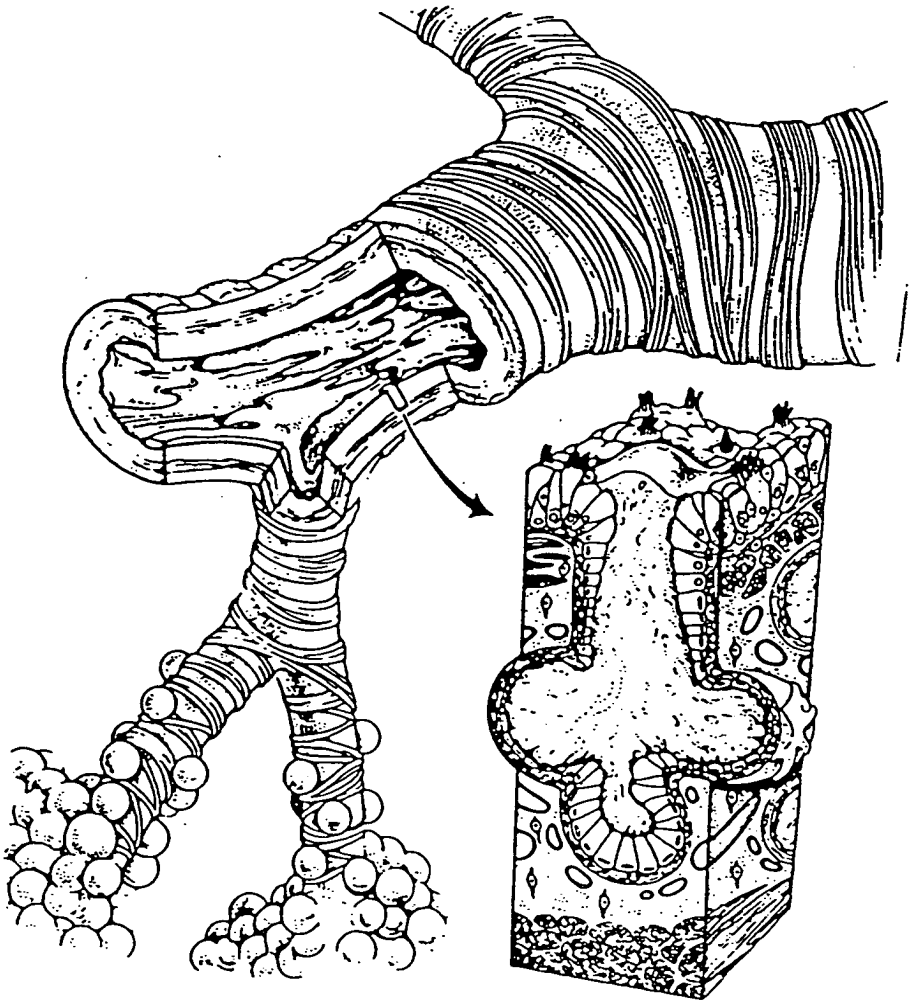


Figure 1.9.5-A. Schematic of the anatomy of the bronchial tree typical for cystic fibrosis in midcourse. The cut-away view shows details of the epithelium and submucosal glands. There are changes to the epithelium with a loss in ciliated cells and an increased number of secretory, basal and undifferentiated columnar cells. The submucosal glands are hypertrophied in some areas and thinned in others. There is thick, purulent mucus in the glands and in the airways.

Table 1.9.5-B

Types of Nasal and Bronchial Epithelial Cells Recovered by Brush of Normals and Individuals with Cystic Fibrosis

Site	Study group	n	Total number of cells recovered per brush	Proportion of Epithelial Cells Recovered[1]			
				Ciliated (%)	Basal (%)	Secretory (%)	Undifferentiated Columnar (%)
Nasal	Normal	25	$1.3\pm0.3\times10^5$	64±3	11±1	15±2	10±1
	CF	12	$1.9\pm0.3\times10^5$	61±3	13±1	15±2	11±1
Bronchial	Normal	70	$4.4\pm0.2\times10^5$	79±1	12±1	4±1	5±1
	CF	22	$10.8\pm1.5\times10^5$	60±2	15±1	8±1	16±1

asal brush, normal vs CF, no difference in the proportions of all categories ($p>0.1$, all comparisons); bronchial brush, normal vs CF, significant difference in the proportion in all categories ($p<0.01$, all comparisons). See reference Trapnell et al, 1991, Danel et al., 1992 for details on methods.

In the final stages of the disease, autopsy studies demonstrate small airways become completely obstructed with secretions. There are bronchiectatic cysts occupying as much as 50% of the cross-sectional area of the lung (Boat et al., 1989) and the bronchioles are stenosed and obliterated. Bronchiectasis with marked derangements of the epithelial surface and the bronchial wall is common.

1.10 CFTR Gene Expression in the Airway Epithelium

The CFTR gene is expressed in the epithelium of the human nose, trachea and bronchi (Trapnell et al., 1991a). On the surface epithelium, expression is very low, averaging 1-2 mRNA transcripts per cell (Trapnell et al., 1991a). Consistent with this observation, the sequence of the 5′ flanking region of the CFTR gene has the characteristics of a housekeeping-type gene, and the rate of transcription of the CFTR gene in normal human bronchial epithelium is only 6% that of the β-actin gene (Chou et al., 1991; Yoshimura et al., 1991b). The absolute level of CFTR gene expression in airway epithelial cells of individuals with CF is not known, but the relative rate of expression of the normal and ΔF508 allele in a heterozygote is equal (Trapnell et al., 1991a). Recent studies suggest normal expression of the CFTR gene may be higher in secretory epithelial cells, particularly serous cells in mucus glands of the large airways (J. Wilson, personal communication). It is not known if this has direct relevance to the pathogenesis of the disease. In mice, there is also expression in the alveolar epithelial cells (J. Whitsett, personal communication), a site where there is no clinical disease (except late in the course of the disease, where there is a large scale derangement of pulmonary architecture).

1.11 Pathogenesis of the Respiratory Manifestations of Cystic Fibrosis

Evidence from a variety of sources strongly argue that the pulmonary manifestations of CF result from abnormal expression of the CFTR gene in epithelial cells for the tracheobronchial tree (Figure 1.11-A). It is not clear, however, how the abnormal expression of the CFTR gene product results in the abnormal mucus, colonization with bacteria and intense, and chronic epithelial inflammation in the lung that characterize the disease. All available evidence is consistent with the concept that it is the inflammation that causes the progressive derangements to the airways that result in respiratory impairment and eventual death from respiratory failure.

1.11.1 CFTR Mutations and Respiratory Disease

In regards to the link of mutations of the CFTR gene to airway disease, there is overwhelming evidence that respiratory manifestations of CF are linked to mutations for the CFTR gene in both parental alleles (Boat et al., 1989). Further, measurements of the transepithelial voltage of the tracheobronchial tree (lumen voltage relative to the submucosa) of CF patients reveal a higher voltage than that observed in normals, or in individuals with other diseases of the tracheobronchial tree, consistent with the concept that there is a local abnormality in electrolyte transport (Knowles et al., 1981). Finally, <u>in vivo</u> evaluation for the airway epithe-

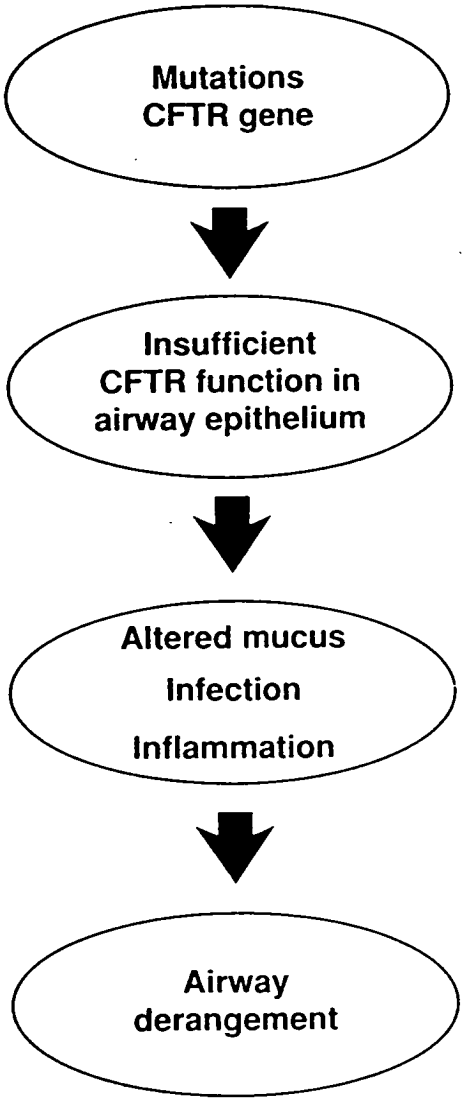


Figure 1.11-A. Pathogenesis of the respiratory manifestations of cystic fibrosis. Consequent to mutations of the CFTR gene there is insufficient CFTR function in airway epithelial cells. Through alterations in the electrolyte milieu of the airway surface and/or abnormal biologic processes within the airway epithelial cells consequent to the insufficient CFTR function, there are alterations in airway mucus, chronic bacterial infection, and an intense, chronic neutrophil-dominated epithelial inflammation. Over time, the inflammation deranges the airways, causing progressive respiratory dysfunction and eventual failure.

lium of individuals with CF receiving lung transplantations demonstrates a normal voltage (Wood et al., 1989).

Primary cultures of airway epithelial cells demonstrate abnormalities in Cl^- permeability and an inability to secrete more Cl^- in response to stimuli that increase intracellular cAMP (Rich et al., 1990) i.e., the epithelial cells of individuals with CF demonstrate an abnormality in regulation of Cl^- transport independent of any systemic factors. Consistent with these observations, permanent airway epithelial cell lines established from CF patients manifests the Cl^- channel abnormality (Jetten et al., 1989; Rich et al., 1990; Wagner et al., 1991; Zeitlin et al., 1991). Single channel patch-clamp studies have demonstrated the Cl^- abnormality is manifest on the apical membrane of airway epithelial cells (Frizzell et al., 1986; Hwang et al., 1989; Li et al., 1988; Li et al., 1989). When the patches were attached to the CF cells, the abnormality in Cl^- permeability was evident. However, when the apical patches' were excised, the Cl^- channel could be made to function i.e., the CF abnormality is directly expressed in airway epithelial cells and appears to involve the regulation of apical Cl^- channels.

Finally, in vitro transfer of the normal human CFTR cDNA into airway epithelial cells from individuals with CF corrects the characteristic abnormality in Cl^- transport (Rich et al., 1990).

1.11.2 How Abnormal CFTR Gene Expression Causes the Characteristic Airway Disease

While the link between mutations in the CFTR gene and the respiratory abnormalities of cystic fibrosis is definitive, the mechanisms involved in the pathogenesis for the airway disease is not. There are two general hypotheses, not mutually exclusive, that explain the process: (1) the respiratory manifestations result from changes in the electrolyte milieu on the surface of the airway epithelium; and (2) the respiratory disease results from processes internal to the respiratory epithelial cells.

Although the exact mechanisms are not defined, an abnormality in electrolyte transport from the apical surface of airway epithelial cells can explain all of the pulmonary manifestations of the disease. In this regard, transepithelial electrolyte transport likely modulates the quantity and composition of respiratory tract epithelial fluid. Thus, an inability to actively transport Cl^- from the submucosa to the epithelial surface in a normal fashion would limit H_2O from being secreted from the epithelium in a normal fashion. Consequently, the airway secretions would be relatively dehydrated, providing a rational scenario for the subsequent development of mucus obstruction, infection and inflammation.

Alternatively, the respiratory disease may result from abnormal expression of the CFTR gene modifying processes internal to the respiratory epithelial cells. While such mechanisms are presently only hypothetical, they might include abnormalities in the pH of intracellular organelles causing dysfunction of enzymes modifying mucins or abnormal expression of cell components that interact with microorganisms such as Pseudomonas.

It is also not known whether all airway epithelial cells are involved in the pathogenesis of the airway disease, or only a subset. For example, if the "external electrolyte milieu" hypothesis is correct, it is likely that most, if not all of the epithelium is involved, consistent with the widespread (albeit low) expression of the CFTR gene in the surface airway epithelium. Alternatively, if the "internal milieu" hypothesis holds, a subset of cells, such as mucus producing cells, may play a dominant role.

Independent of the mechanisms leading to the abnormal mucus, bacterial colonization and inflammation, there is general agreement that the intense neutrophil-dominated inflammation deranges the airway epithelium (Doring, 1989; McElvaney et al., 1991). While a variety of mediators are likely involved, injury to the epithelium is mediated to a large extent by neutrophil elastase and neutrophil-generated oxidants (McElvaney et al., 1991; McElvaney et al., 1992; Roum et al., 1990).

1.12 Rationale for Using a Replication Deficient Adenovirus Containing the Human CFTR cDNA to Treat the Respiratory Manifestations of Cystic Fibrosis.

Although the pathogenic processes causing the pulmonary manifestations of CF are not exactly identified, the available evidence presents an overwhelming case for the fundamental abnormality in the lung of CF patients to be an abnormality in expression of the CFTR gene in the airway epithelium. This strongly supports the concept that it is rational to use gene transfer directly to airway epithelial cells to correct the CF defect that ultimately causes the major clinical manifestation of the disease.

One strategy to accomplish this is to utilize a replication deficient recombinant adenovirus that contains an active promoter and a normal CFTR cDNA (Rosenfeld et al., 1992). In addition to the ability to accommodate a large (up to 7.5 kb) exogenous cDNA, the adenovirus has the advantage of being tropic for respiratory epithelium and capable of transferring recombinant genes into non-proliferating cells. The recombinant adenoviral approach has been successful in transferring the human α1-antitrypsin gene to the respiratory epithelium of experimental animals in vivo (Rosenfeld et al., 1991) and directly relevant to this protocol, a recombinant adenovirus containing a normal CFTR cDNA has been successful in mediating in vivo transfer and expression of the human CFTR gene to the respiratory epithelium of the lungs of experimental animals (Appendix 1). Further, a recombinant adenovirus containing the human CFTR cDNA will correct the Cl^- secretory abnormality of epithelial cells of individuals with CF in vitro. Details of the vector are presented in Section 2 and details of the experimental evidence supporting the use of a replication deficient recombinant adenovirus containing the human CFTR cDNA to treat the respiratory manifestations of cystic fibrosis are presented in Section 3.

In contrast to the adenovirus, there is currently no other vector system capable of transferring the CFTR cDNA to the airway epithelium in vivo with efficacy and efficiency necessary to treat the disease. The only other vector system that has been shown to mediate in vivo transfer of the human

CFTR cDNA to the respiratory epithelium in vivo in experimental animals are double stranded DNA circular DNA plasmids combined with liposomes (Yoshimura et al., 1992). However, the extent of expression with this vector system is very limited, with no possibilities of therapeutic benefit to the individual. Further, this limited expression can be achieved only with very large amounts of DNA (equivalent to 500 mg for a 50 kg human). Of the other vector systems considered (retrovirus, adeno-associated virus, naked plasmid, plasmid linked to ligand, plasmid delivered in association with adenovirus or fragments of adenovirus, vaccinia, or influenza), there is currently no in vivo evidence that any will work in the required fashion.

SECTION 2

REPLICATION DEFICIENT RECOMBINANT ADENOVIRUS CONTAINING THE HUMAN CFTR cDNA

2. Replication Deficient Recombinant Adenovirus Containing the Human CFTR cDNA

The two vectors evaluated in this protocol, AdCFTR and Av1CF1, are identical in general construction, differing only in the "expression cassette" relating to control of expression of the CFTR cDNA (see Figures 2.3-A, 2.3-B and Table 2.3-B, in Section 2.3 below). AdCFTR was constructed by Transgene SA, Strasbourg, France and M. Perricaudet (CNRS), Institut Gustave Roussy, Villejuif, France (see Appendix 1). Av1CF1 was constructed by B. Trapnell, M. Rosenfeld and R. Crystal, in the Pulmonary Branch, NHLBI. Both are based on the genome of Ad5, a member of the common subgroup C of adenoviruses (see Section 2.1, below). In both, all of the Ela region has been deleted, as have the 69.5% of the left hand portion of Elb and 66% of the middle part of the E3 region. Both vectors begin (starting from the left end) with the left inverted terminal repeat (ITR) and origin of replication from Ad5 and the encapsidation signal and Ela promoter/enhancer element from Ad5. In both vectors, this is followed by the major late promoter (MLP) and tripartite leader sequences; in AdCFTR these elements are from Ad2 (also a member of subgroup C, with 94.7% sequence homology to Ad5), while in Av1CF1 they are from Ad5. Following the tripartite leader, both contain the normal human CFTR cDNA flanked by short 5' and 3' untranslated regions from the CFTR genome; there are minor differences in the two vectors in the length of these regions and the sequence of the CFTR cDNA (see Table 2.3-B). The major difference in the two vectors is following the CFTR cDNA 3' untranslated region. In AdCFTR there is a poly A signal (the SV40 early region poly A signal) whereas this element is not present in Av1CF1 (Av1CF1 uses a poly A signal in the Elb region, see Figure 2.3-A). Following the CFTR cDNA "expression cassette", AdCFTR and Av1CF1 are identical and are based on the sequences of Ad5 from 9.24 m.u. through the right ITR. Both have two deletions in this sequence (see Appendix 3): (1) a 2 bp deletion in the VA-I promoter that interrupts a minor alternative transcription start site for VA-I RNA; and (2) a deletion of the 66% of middle part of 3. Av1CF1 and AdCFTR were derived from different base Ad5 deletion mutants (Addl327 for Av1CF1, Addl324 for AdCFTR); this difference is irrelevant to the final construct, with the right end of AdCFTR and Av1CF1 identified after the CFTR expression cassette. For a discussion of the derivation and final structure of Av1CF1, see Section 2.1, and appendix 3. For a discussion of the derivation and final structure of AdCFTR, see appendix 1 and appendix 3. The recombinant genomes of both AdCFTR and Av1CF1 are packaged into replication deficient recombinant adenovirus using 293 cells (Graham et al., 1977), a human embryonic kidney cell line containing El sequences of Ad5.

Both AdCFTR and Av1CF1 will infect human epithelial cells and express the human CFTR cDNA as evidenced at the mRNA level (Northern analysis) and protein level (immunohistochemistry, immunoprecipitation followed by phosphorylation with kinase). For both vectors, the expression of the CFTR cDNA is in amounts sufficient to complement mutations of the CFTR gene, enabling the epithelial cells derived from individuals with CF to secrete Cl^- in response to elevations in cAMP. In the sections dealing with efficacy (Section 3) and safety (Section 4) data with both vectors are presented; for all of the data presented, the specific vector used (AdCFTR or Av1CF1)

is indicated. Although both vectors function to complement mutations of the CFTR gene in epithelial cells derived from individuals with CF in an appropriate fashion, direct comparisons of the two vectors with Northern, immunohistochemistry, immunoprecipitation followed by phosphorylation with kinase and functional assays (ability to secrete Cl^- in response to elevations of cAMP) demonstrated that AdCFTR consistently yielded more expression/function per pfu. Based on this analysis, a decision was made to use AdCFTR for the clinical protocol.

The following sections provide an overview of the structure of the adenovirus, the fabrication and production of the recombinant adenoviruses AdCFTR and AvlCFl, the structure of AdCFTR and AvlCFl, formulation of the recombinant vector for the clinical protocol, and the various quality control parameters to assess the recombinant virus to be used in the clinical protocol.

2.1 Overview of the Adenovirus

The adenovirus is comprised of linear, double stranded DNA complexed with core proteins and surrounded with capsid proteins. The intact virion has icosahedral symmetry (20 plane faces, 12 vertices), a molecular mass of 175-185 x 10^6 Da, and a diameter of 88 nm (see Ginsberg, 1984; Horwitz, 1990a; Berkner, 1988 for an overview of adenoviruses; Stewart et al., 1991 for the 3-dimensional structure). The 36 kb of double stranded DNA comprises 11.6-13.5% (by weight) of the virus. The remainder is protein, except for approximately 1% carbohydrate. There is no lipid. The virion sediments at 31-32S and has a buoyant density in CsCl of 1.33 - 1.35 g/ml.

There are 47 distinct serotypes of adenoviruses and several additional viruses being considered for classification (Hierholzer, 1992). The clinical illnesses associated with adenovirus infection differ among groups of serotypes. The illnesses are usually mild but on rare occasions can be life threatening. The common illnesses include acute febrile pharyngitis, kerato-conjunctivitis, and gastroenteritis. Less commonly, adenovirus infection is associated with bronchitis, pneumonia, a pertussis-like syndrome, acute hemorrhagic cystitis, and hepatitis. Adenovirus is widespread in the general population, with evidence of anti-adenovirus antibodies in most adults. For example, in 1954 in Washington, D.C., >95% of individuals surveyed had serologic evidence of prior adenovirus infection by ages 16-34 years (Straus, 1984).

The AdCFTR and AvlCFl vectors to be used in this protocol are based on adenovirus type 5, a common serotype belonging to subgroup C, a subgroup that includes serotypes 1, 2, 5, and 6 and is variably associated with malaise, fever, chills, myalgia, headache, rhinorrhea, nasal congestion, sneezing, anorexia, conjunctivitis and pharyngitis (Horwitz, 1990a; Straus, 1984). A 10 year study in Washington, D.C. revealed two annual peaks in adenovirus-associated respiratory illness, July and December (Straus, 1984). Following respiratory adenovirus infection, shedding is observed from the respiratory tract, but fecal shedding continues for a longer period (Fox et al., 1969). Adenovirus-associated respiratory disease can be

transmitted by the respiratory, oral, or conjunctival routes. The incubation period ranges from 5-10 days with an average of 7 days.

The immune/inflammatory system contains and clears adenovirus infections through a variety of interactions involving humoral and cellular processes. Although humoral responses can modify the course of adenovirus infection, it is clear that cell-mediated immune mechanisms are central to the containment and resolution of these infections (Straus, 1984). Lower respiratory tract infection with frank pneumonia is uncommon with adenovirus infection; when it does occur in adults it is almost always associated with adenovirus subtypes 3, 4, 7, and 21, not 5. There are rare fatalities with adenoviral infection; almost all cases of devastating adenovirus infections have been documented in circumstances in which there is inadequate cellular immunity, including neonates, immune-depressed individuals with cancer, transplants, HIV infection, and individuals receiving corticosteroids, cytotoxic drugs or radiation, and children with T-cell disorders (Horwitz, 1990a; Straus, 1984; Hierholzer, 1992). There is no evidence that children or adults with CF have increased susceptibility to or severity of adenovirus infection.

Despite the fact that all subgroups of adenovirus can transform cells from various species in vitro and some subgroups (A and B) can induce tumors in newborn hamsters, there is no evidence that any adenovirus type is associated with human tumors in vivo (Horwitz, 1990a; Straus, 1984). The vectors AdCFTR and Av1CF1 are based on adenovirus type 5, a member of subgroup C, a subgroup that does not produce tumors in any animal in vivo (Horwitz, 1990a; Straus, 1984).

Because of the widespread prevalence of adenovirus infection in closed populations such as military recruits, considerable effort was put into developing adenovirus vaccines. In 1963, live adenovirus vaccines were developed using enteric-coated capsules containing adenovirus types 4 and 7 (Chanock et al., 1966; Edmondson et al., 1966; Gutekunst et al., 1967; Smith et al., 1970; Top et al., 1971b; Top et al., 1971c). These vaccines cause inapparent enteric infection. There is no spread of infection to the respiratory tract or evidence of respiratory symptoms. Spread to non-infected individuals is rare. The oral vaccines elicit serum but not local, neutralizing antibodies on the nasal epithelial surface (Scott, 1972; Smith, 1970). Since the initial development of these vaccines, their use is widespread in the military, with bivalent (Ad4, Ad7) live enteric vaccines in general use in the US and other militaries around the world. It is estimated that >5 million individuals have had live adenoviruses administered in this form (Chanock et al., 1966; Couch et al., 1963; A. Davis, personal communication; Top et al., 1971b; Top et al., 1971c).

2.2 Structure of the Adenovirus

The structure of the adenovirus is conveniently described on the basis of the adenovirus genes expressed following infection of human cells. By convention, the 36 kb of adenovirus DNA is divided into 100 map units (mu); 360 bp/mu. The viral genome is transcribed in two major stages, an early (E) phase which precedes viral DNA replication and a late (L) phase (start-

ing 6-8 hours later) (Figure 2.2-A). The early phase genes are grouped as E1a, E1b, E2a, E2b, E3 and E4. Part of the L1 region is also grouped as an "early" phase gene. Regions active in both the early and late phase include E2a, E2b, and IX. The late phase genes are grouped as part of L1, L2, L3, L4, and L5. Also in the late phase there is transcription of regions IVa2 and VA.

The early region genes code for at least 30 mRNA transcripts. Specific functions have been defined for many, but not all of the proteins coded by these transcripts (see Pettersson, 1984 for details). The E1 products are the first adenovirus proteins generated post-infection and function as important transcriptional regulators. Importantly, most of the E1 region sequences are critical for viral replication; by removing the E1a region and a substantial part of E1b, the virus can be rendered replication deficient. The E3 region can be deleted with no apparent effect on viral growth in culture (see section 4.8 for a discussion of this region and the safety aspects of deleting E3 sequences).

The late region genes are mostly related to production of the structural proteins required for assembly of the nucleoprotein core (e.g. the genomic DNA of the virus plus the proteins associated with the DNA) and the capsid forming the outer shell of the virus (Ginsberg, 1984; Horwitz, 1990a). The five families of late mRNAs (L1-5) all use the same tripartite leader sequences 1, 2, and 3 found 5′ to the L region genes (Figure 2.2-A).

Besides the early and late regions genes, other important regions of the adenovirus genome include the left and right inverted terminal repeats (ITR), and sequences required for initiating replication and encapsidation (ψ) of the virus.

2.2.1 Structural Proteins

Each adenovirus virion has approximately 2,700 polypeptides. The vast majority of these are the structural proteins, most of which comprise either the capsid or are associated with both the capsid and the nucleoprotein core (Table 2.2.1-A). Analysis of the purified adenovirus AdCFTR by sodium dodecyl sulfate acrylamide gels reveals the same major structural proteins found in replication competent Ad5 (P. Seth, R. Crystal, not shown). For details as to the structure, location, function and assembly of these proteins, see Pettersson, 1984.

2.3 Structure of the Replication Deficient, Recombinant Adenovirus Vectors

The replication deficient, recombinant adenovirus vector Av1CF1 is based on the genome of human adenovirus type 5. The naturally occurring Ad5 has a double stranded DNA genome of 35,935 bp (GenBank accession No. M73260). The overall structure of AdCFTR has been published (see Appendix 1). Av1CF1 has a genome of 36,397 bp. As with AdCFTR, Av1CF1 has two major structural deletions (relative to Ad5), an E1 deletion (1.26-9.24 mu of Ad5) and an E3 deletion (78.5-84.7 mu of Ad5). Also idential to AdCFTR, Av1CF1 has a minor 2 bp deletion in the VA-I promoter (see appendix 3). The overall structure

Figure 2.2-A. Transcription and translation maps of adenovirus type 2. Ad2 and Ad5 (the base virus for AdCFTR) belong to the same subgroup and have 94.7% nucleic acid sequence identify. The left strand (5′ to 3′) and right strand (3′ to 5′) are divided into 100 map units (m.u.). The early mRNAs (E1a, E1b, some of E2A, most of E2B, E3 and E4) are indicated as thick arrows. The intermediate mRNAs expressed early and late (some E2a, some E2b and IX) are indicated as thin arrows. The late mRNAs (L1, L2, L3, L4, and L5) are indicated as open arrows. The direction of transcription is indicated by the arrows. The capped 5′ ends of the mRNA (indicated by a bracket to the left or right of the start of the arrow) indicate the position of the transcriptional promoters, and the end of the arrowheads indicate the 3′ ends of the mRNA. Gaps indicate intervening sequences removed by splicing to form the mature cytoplasmic mRNA. The L1-L5 mRNAs all originate with the same tripartite leader sequences 1, 2, and 3 (except for one L1 mRNA which uses the fourth leader segment "i"). Also indicated are most of the structural and nonstructural proteins comprising the virion. For further details see Berkner, 1988; Ginsberg, 1984; Horwitz, 1990a.

Figure 2.2-A

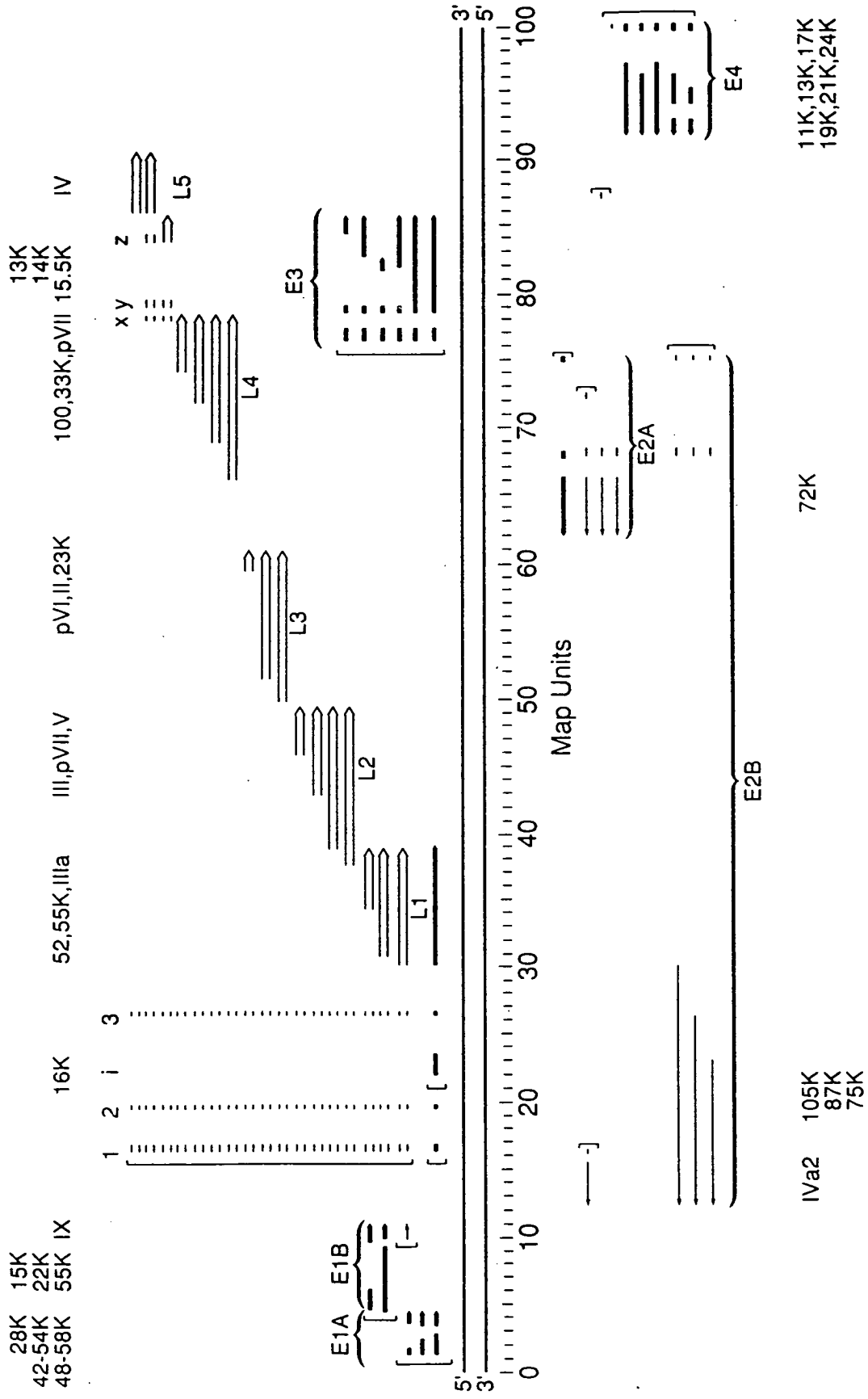

28K
42-54K
48-58K
15K
22K
55K
IX
E1A
E1B
16K
1
2
i
3
52,55K,IIIa
III,pVII,V
pVI,II,23K
100,33K,pVII
13K
14K
15.5K
IV
x y
z
L1
L2
L3
L4
L5
E3
E2A
E2B
E4
5'
3'
0
10
20
30
40
50
60
70
80
90
100
Map Units
IVa2
105K
87K
75K
72K
11K,13K,17K
19K,21K,24K

Table 2.2.1-A

Structural Proteins of the Adenovirus[1]

Category	Component	Polypeptide	MW (kDa)	Native form of molecule in Ad	MW of native form (kDa)	# of native molecules per virion	# of polypeptides per virion	Transcription unit coding for polypeptide	Notes
Major surface proteins	Hexon	II	108	trimer	324	240	720	L3	
	Penton base[2]	III	85	trimer	246-256	12	36	L2	
	Fiber[2]	IV	62	trimer	156-207	12	36	L5	
Major core proteins	Core protein	V	48	monomer	47	180	180	L2	
	Core protein	VII	18.5	monomer-tetramer	18-83	1070	?	L2	
Minor polypeptides	Vertex region	IIIa	66	monomer	65	60	60	L1	IIIa is phosphorylated[4]; associated with hexon polypeptide
	Hexon - associated	VI	24	dimer or trimer	50-72	450	900 or 1350	L3	
	Hexon - associated	VIII	14	monomer	13	127	127	L4	
	Hexon nonomer - associated	IX	13	dimer	23	240	560	E1b[3]	Associated with "nonomer" of hexons of each face
		X-XII	5-6.5						Three different minor polypeptides; relationship to other proteins unknown
		μ	4-5			125			May be identical to X,XI or XII

[1] Based primarily on Ad 2; referred to as "structural" proteins because they are identified on SDS - polyacrylamide gels of purified Ad; for additional details, see Pettersson, 1984.
[2] Penton base + fiber is called "penton" (MW 365 kDa).
[3] IX is different from other Ad structural proteins in that the transciptional unit coding for it (E1b) is expressed at intermediate as well as late times after infection with wild type virus.
[4] In addition to IIIa, there is evidence that V, VI, VII and X may also be phosphorylated, but to a much lesser degree.

of AdCFTR and AvlCF1 compared to the genome of Ad5 is shown in Figure 2.3-A and details of the "expression cassette" of both vectors is shown in Figures 2.3-B. In both vectors, the E1 deletion encompasses the entire E1a coding region and 69.5% of the left hand end of the E1b region. The E3 deletion in both vectors comprises 66% of the E3 region (leaving 20% of the left end of E3 and 14% of the right end of E3). AdCFTR and AV1CF1 have minor differences in regards to the "expression cassette" relating to the expression of the CFTR cDNA. These are detailed in Section 2., above and in Table 2.3-B. The sequence of AvlCF1 (based on direct sequencing of the expression cassette and the published sequence of Ad5) is in appendix 3. The minor sequence differences of AdCFTR from this sequence are detailed in Table 2.3-B. These regions of minor differences of AdCFTR as compared to AvlCF1 in the "expression cassette" [the left hand end through the beginning region of homology with the regions of minor differences of Ad5 (9.24 mu)] are being sequenced and will be made available to the Recombinant DNA Advisory Committee and the FDA.

The deletions of Ad5 used as the base for AdCFTR total 4754 bp and AvlCF1 total 4743 bp. To make AdCFTR amd AvlCF1, the expression cassettes detailed in Figures 2.3-B and Table 2.3-A were combined by homologous recombination with the $E1^{-}E3^{-}$ Ad5 base to yield a final genome of approximately 101 mu. The upper limit of genomic DNA that can be packaged into an infectious Ad virion is 105 mu. At 101 mu, AvlCF1 and AdCFTR are approximately 3.8% less than the maximal packagable genome.

From a functional point of view, AdCFTR and AvlCF1 are replication deficient since both are missing E1 genes coding for E1a proteins [28K, 42-54K, and 48-58K proteins] and E1b proteins [15K, 22K and 55K proteins] that play a critical role in regulating replication. Given that these vectors are replication deficient and missing most of E3, they should not have the ability of Ad5 to subvert the immune/inflammatory system (See section 4.8). From the newly inserted information, AdCFTR and AvlCF1 contain elements to direct the expression of the human CFTR cDNA [the E1a promoter/enhancer, the MLP and the tripartite leaders (sequences that increase translation efficiency)]. This is followed by the CFTR cDNA itself. The CFTR mRNA directed by AdCFTR likely uses the SV40 early region polyadenylation signal 3' to the CFTR 3' untranslated region, while the CFTR mRNA directed by AvlCF1 likely uses the polyadenylation signal within the Ad5 E1b region (4038 to 4043 bp of Ad5).

The packaged AdCFTR and AvlCF1 virions have a CsCl density similar to Ad5 (1.33-1.35 g/ml).

2.4 Fabrication and Production

AdCFTR and AvlCF1 were constructed by homologous recombination of an adenovirus vector construction plasmid containing the human CFTR cDNA and Ad5 sequences and the large ClaI fragment of Add1327 (for AvlCF1, a replication competent adenovirus type 5 based $E3^{-}$ mutant) or Add1324 (for AdCFTR, a replication deficient adenovirus type 5 based $E1^{-}E3^{-}$ mutant). The regions of Add1327 and Add1324 that are in AvlCF1 and AdCFTR, respectively, have identical sequences (Add1327 was derived from Add1324; T. Shenk, personal

Figure 2.3-A. Genomic organization of Av1CF1 and AdCFTR compared to Ad5. Shown (top to bottom) are a map of the Ad5 genome indicating the regions deleted in Av1CF1 and AdCFTR and overall maps of Av1CF1 and AdCFTR. The regions of Ad5 not present in Av1CF1 and AdCFTR include the entirety of E1a and 69.5% of the left end of E1b and 66% of E3. The deletion of the E1 region is replaced with the Ad5 major late promoter (MLP; from Ad5 in Av1CF1 and from Ad2 in AdCFTR), the Ad tripartite leader sequences 1, 2, and 3 (from Ad5 in Av1CF1 and from Ad2 in AdCFTR)and the human CFTR cDNA. AdCFTR includes an SV40-derived polyA signal after the CFTR cDNA; Av1CF1 uses the E1b polyA signal (indicated). ITR = inverted terminal repeat. Not shown - the minor 2 bp deletion of the VA-I promoter (in the region of 29 m.u.) in both Av1CF1 and AdCFTR.

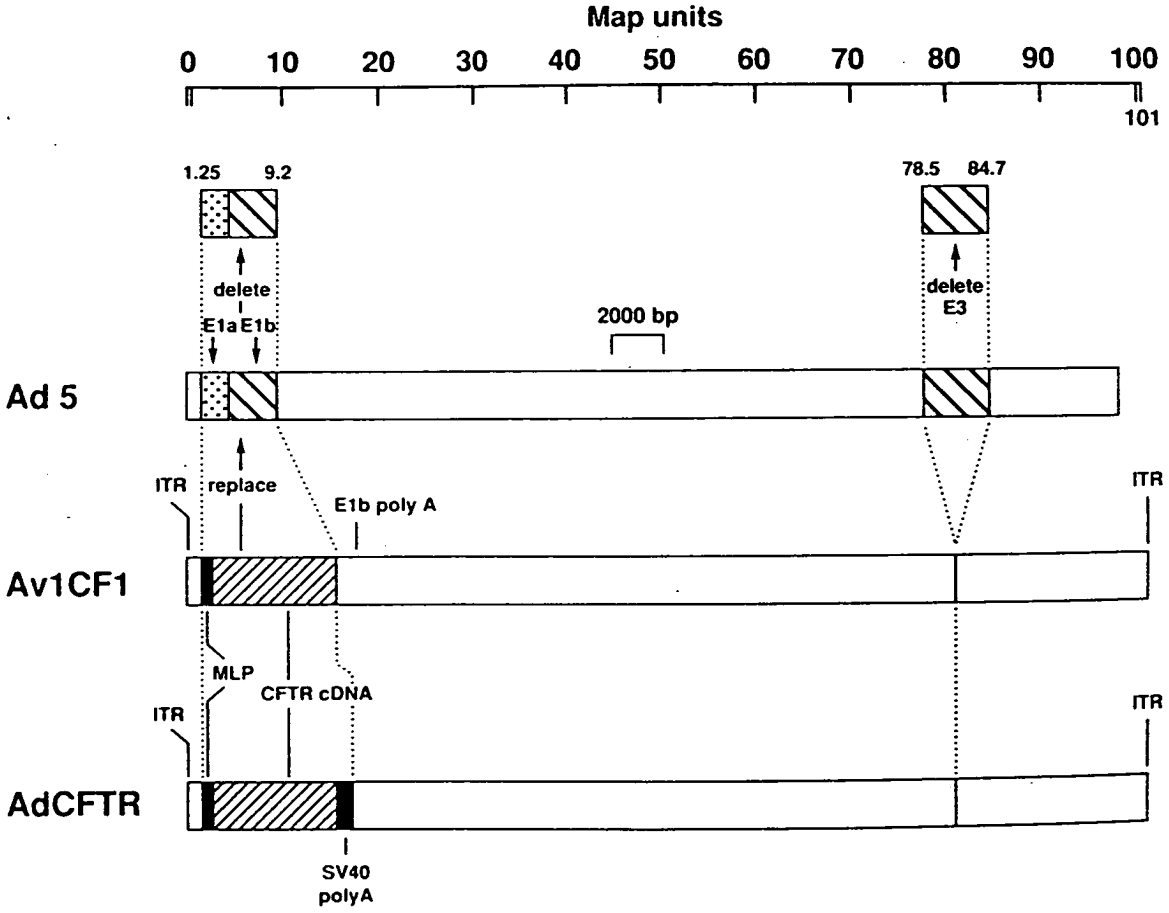


Figure 2.3-B. The left hand end "expression cassette" of AdCFTR compared to Av1CF1. ITR = inverted terminal repeat; the scale is in basepairs (bp); to demonstrate all elements (e.g., linking sequences), the schematics are not exactly to scale. See section 2.3 and Table 2.3-B for details.

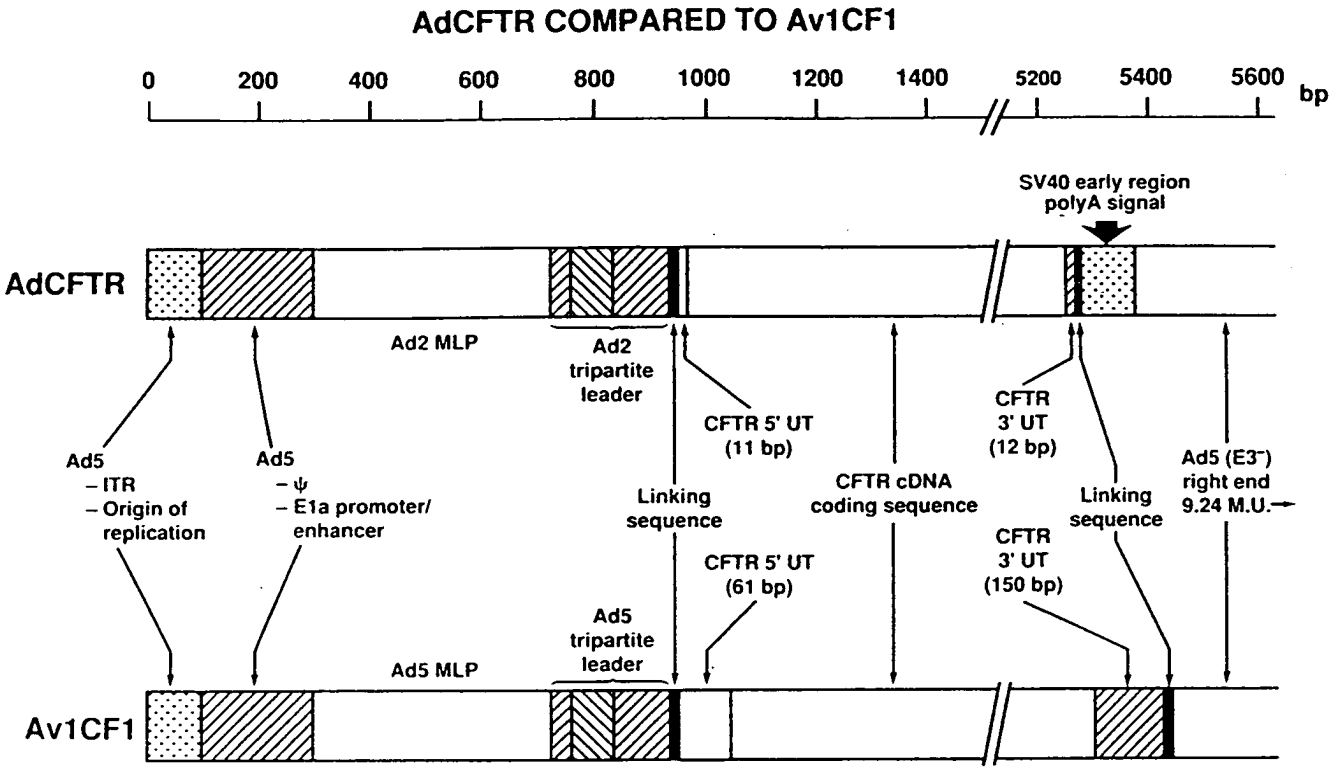


Table 2.3-B

Structural Elements Contained in the Genome of AdCFTR and Av1CF1

Element	AdCFTR	Av1CF1	Differences
ITR origin of Replication	Ad5	Ad5	None
ψ + E1 promoter/ enhancer	Ad5	Ad5	Av1CF1 includes 11 bp more of the E1 promoter[1]
Major late promoter	Ad2	Ad5	2 bp[2]
Tripartite leader	Ad2	Ad5	Minor sequence differences in 3rd tripartite leader[2]
CFTR gene 5' untranslated region	11 bp	61 bp	AdCFTR contains less 5' untranslated region[3]
CFTR cDNA coding sequence	Normal	Normal	Minor differences[3]
CFTR gene 3' untranslated region	12 bp	150 bp	AdCFTR contains less 3' untranslated region[3]
SV40 early region polyA signal	Yes	No	AdCFTR but not Av1CF1 contains SV40 polyA signal[4]
Ad5 right end[5]	From 9.24 m.u. to right end[6]	From 9.24 m.u.to right end[6]	None

[1]Ad5 E1a promoter sequences 455-466 are included in Av1CF1, not in AdCFTR

[2]GenBank accession #M73260

[3]For AdCFTR see Appendix 1 and Yoshimura et al., 1991b; for Av1CF1 see Appendix 3

[4]SV40 early region 2666-2538 bp (GenBank accession # V01380)

[5]Most of E3 region deleted and a 2 bp deletion of the VA-I promoter, see section 2.3

[6]Appendix 3

communication). The details of the fabrication of AdCFTR have been published (see appendix 1). The fabrication of AvlCF1 is described in Figures 2.4-A, 2.4-B and 2.4-C. The plasmid pS2CFTR (Figure 2.4-B) was constructed by combining the base shuttle plasmid pS2 (Figure 2.4-A) with the entire coding sequence of the CFTR cDNA contained within the plasmid pBQ4.7 (Riordan et al., 1989; GenBank accession No. M28668; L-C Tsui, personal communication). The final genome of AvlCF1 includes (left to right) sequences from pS2CFTR (1-5667 bp in AvlCF1) and sequences from Ad-dl327 (5668-38,397 bp in AvlCF1). The pS2CFTR derived sequences provide AvlCF1 with the left ITR, origin of replication, packaging signal (ψ), Ela promoter/enhancer element, major late promoter and tripartite leader, and the CFTR cDNA. The Ad-dl327 derived sequences provide AvlCF1 with all of the necessary remaining elements (except for the products of the deleted El sequences which are provided in trans by the 293 cells used for production, see below) to produce a replication deficient, recombinant infectious virus.

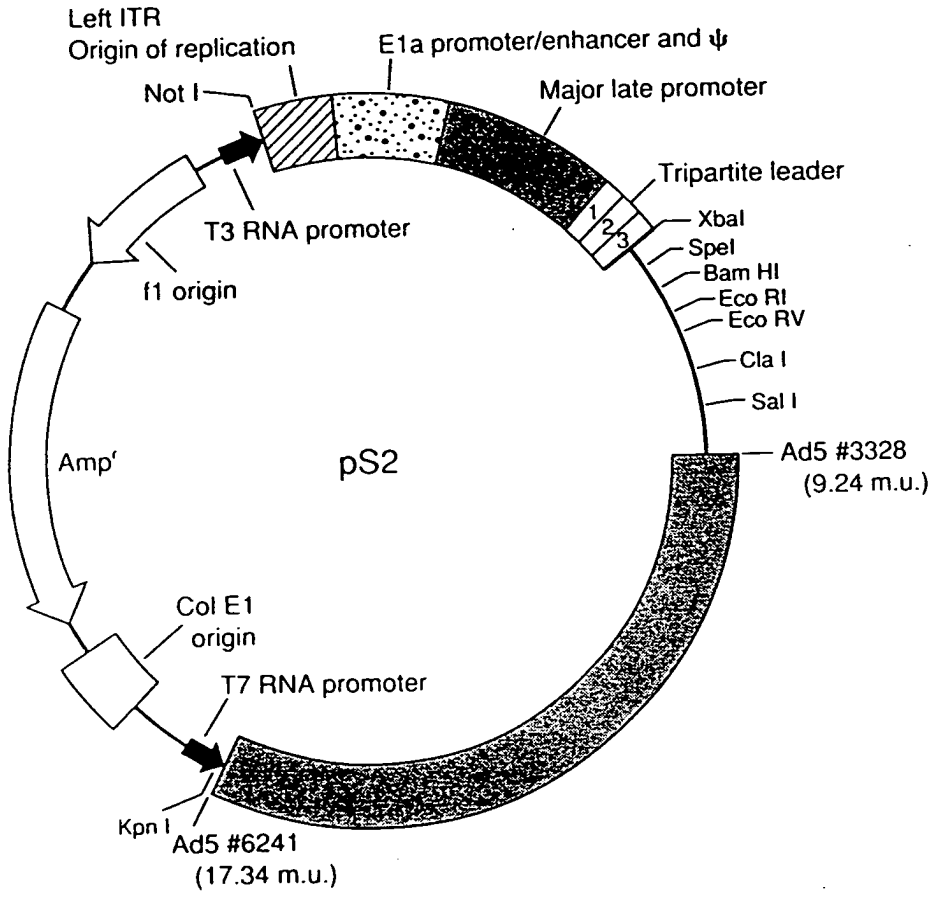
The base shuttle plasmid pS2 was constructed using adenovirus sequences derived from Ad-dl327, a mutant adenovirus identical to Ad5 except for the minor 2 bp deletion in the VA-I promoter and a deletion of the viral genome within the E3 region (sequences 78.5 to 84.7 m.u. are deleted, see Figure 2.4-C). The Ad-dl327 sequences in pS2 are the left hand end of Ad-dl327 (0-1.25 m.u.) including the left hand ITR, origin of replication packaging signal (ψ) and Ela promoter/enhancer. This is followed (left to right) by the MLP (16.1 to 16.8 m.u. in Ad-dl327) and the tripartite leader sequences from Ad-dl327 (16.8 to 16.9, 19.8 to 19.9 and 26.8 to 27.0 m.u. in Ad-dl327). Each of these elements from Ad-dl327 were prepared using PCR amplification of Ad-dl327 DNA as a template. The combined elements from Ad-dl327 were cloned as a NotI-XbaI fragment to a pSKII$^+$ derived plasmid containing a 2913 bp BglII - HindIII fragment of Ad-dl327 (sequences from position 3328 to 6241 of Ad-dl327, identical to positions 3328 to 6241 of Ad5 (see Appendix 3) which had been cloned as a blunt fragment into the XhoI site of the pSKII$^+$ plasmid (GenBank accession No. 52328).

The plasmid pBQ4.7 includes a PstI insert of 4.7 kb encompassing the entire coding region of the CFTR cDNA and flanking sequences beginning with the 5' untranslated sequence at position 72 and ending with the 3' untranslated sequence at position 4725 (see GenBank accession No. M28668 for numbering). The CFTR cDNA with these flanking sequences were removed from this plasmid by cleavage with SpeI and ClaI and cloned into pS2 (cut with SpeI and ClaI) to form pS2CFTR (Figure 2.4-B).

For the final construction of AvlCF1, DNA was purified from Ad-dl327 by proteinase K digestion, phenol chloroform extraction and dialysis against 10 mM Tris-Cl, 1 mM EDTA, pH 7.4. The DNA was cleaved with ClaI (Figure 2.4-C), fractionated by agarose gel electrophoresis, and the large (35 kb) fragment purified by phenol chloroform extraction and dialysis. The purified large ClaI fragment of Ad-dl327 (5 μg) and linear NotI-KpnI pS2CFTR DNA were contransfected with $CaPO_4$ into 293 cells [50% confluent, 6 cm plate].

293 cells are used for the production of both AdCFTR and AvlCF1. 293 cells

Figure 2.4-A. Schematic of the adenoviral construction base plasmid, pS2. In addition to prokaryotic elements including an ampicillin resistance gene (AMP^r), and f1 and Col E1 origins of replication and the T3 and T7 RNA promoters, the plasmid contains left end elements of adenovirus type 5 including the left inverted terminal repeat (ITR) and origin of replication, Ela enhancer and encapsidation signal (ψ), and elements to control the expression of an exogenous cDNA including the major late promoter (MLP) and tripartite leader elements 1,2 and 3. This is followed by a multiple cloning site and a segment of adenovirus type 5 [from position 9.24 map units (m.u.) to 17.34 m.u. (6241 bp)] used for vector construction by homologous recombination.



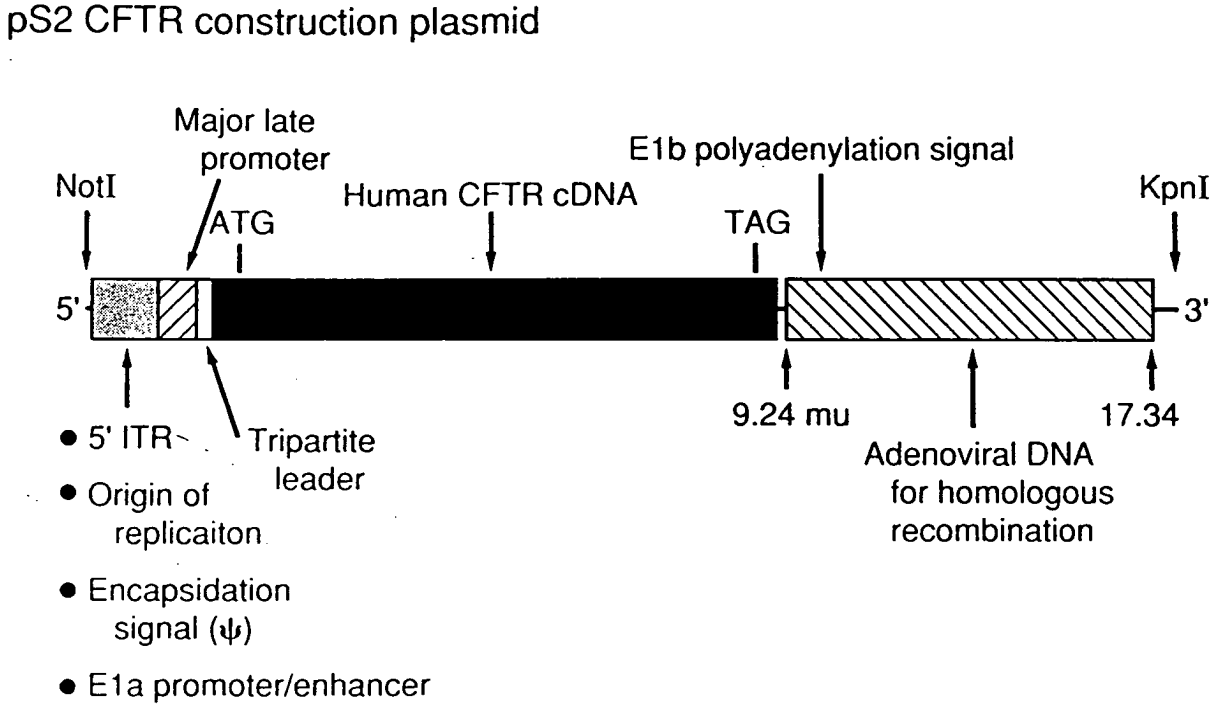
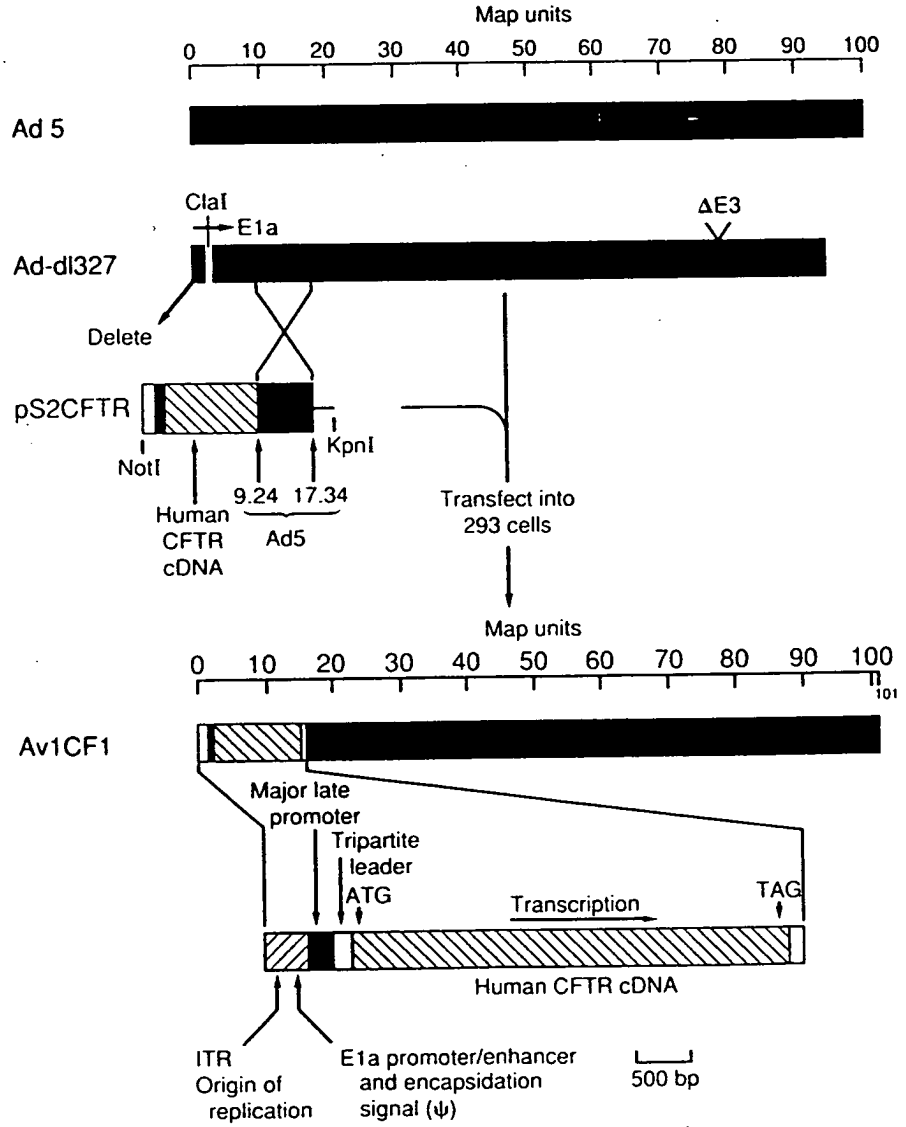


Figure 2.4-B. Schematic of the adenoviral construction plasmid pS2CFTR used in the final construction of AvlCFl. pS2CFTR was constructed by inserting the CFTR cDNA into the base plasmid pS2 (see Figure 2.4-A). Shown are left end genomic elements of Ad5 including the left inverted terminal repeat (ITR) and origin of replication. This is followed by the Ela enchancer and encapsidation signal (ψ), the major late promoter, tripartite leader, and a segment of adenovirus type 5 [from position 9.24 m.u. (3328 bp) to 17.34 m.u. (6241 bp)] used for homologous recombination.

Figure 2.4-C. Construction of Av1CF1 (a recombinant adenovirus expressing human CFTR used for transferring the CFTR cDNA) by homologous recombination of the E3 deletion mutant Ad-dl327 and plasmid pS2CFTR. The majority of the E3 region has been deleted in Ad-dl327; there is also a minor 2 bp deletion of the VA-I promoter (region of 29 m.u.; not shown). The site of the Ela region is indicated by the horizontal arrow. Within the site is a convenient ClaI site. Ad-dl327 is digested with ClaI to yield the "ClaI large fragment". The shuttle plasmid pS2CFTR is cleaved with NotI and KpnI and co-transfected with the ClaI large fragment into 293 cells where homologous recombination occurs to yield the infectious deficient recombinant adenovirus Av1CF1. At the bottom is shown Av1CF1 (total length 101 m.u.) with an enlarged view of the left end. These elements are identical to those in pS2CFTR, see Figure 2.4-B.



are human embryonic kidney cells that have been transformed into a continuous line by Ad5; the genome of 293 cells include 11% of the left end of the Ad5 genome (0 to 11 m.u.) (Graham et al., 1977; Precious and Russell, 1985). This region provides E1 functions in trans that have been deleted from the genome of AdCFTR and Av1CF1, and permits production of replication deficient, recombinant infectious virions of AdCFTR and Av1CF1. The 293 cells were transfected with the purified ClaI cut genome from Addl 327 together with the plasmid DNA for AdCFTR or Av1CF1 and then cultured in a humidified atmosphere at 37° with 5% CO_2. After 18 hours, the cells were washed and 3 ml of soft agarose media (minimal essential media containing 1% seaplaque agarose and 7.5% fetal calf serum) was added. Plaques were picked after 15 days and amplified on 293 cells in successively larger plates usirg standard methods (Green et al., 1967; see Appendix 1 for further details).

High titers of the AdCFTR and Av1CF1 recombinant adenovirus vector were produced in 293 cells and purified on cesium chloride gradients. To accomplish this, cells were grown to 70-80% confluency and infected with purified AdCFTR or Av1CF1 virus at a multiplicity of infection of 10 pfu/cell and cultured until the cells showed typical cytopathic effect. The cells were then harvested and lysed by freezing and thawing to release intracellular virus. The crude viral lysate was cleared of cellular debris by low speed centrifugation. Infectious virus was then recovered by centrifugation on a two step cesium chloride gradient to remove low molecular weight cytoplasmic contents and empty viral capsids (Precious and Russell, 1985). The virus was further purified by isopycnic density centrifugation on cesium chloride. Cesium chloride was removed by dialysis and the resultant virus was aliquoted and stored at -70° until use. The final viral preparation included 10% glycerol to stabilize the virus during storage.

The amount of infectious viral particles in all preparations was determined by plaque assay to titer adenovirus (Green et al., 1967). The plaque assay was carried out with serial dilutions of the virus used to infect 75% confluent 293 cells cultured in 6 well plates. After 90 minutes, the media was changed to soft agarose media and the cultures were continued in a humidified environment at 37° in 5% CO_2 and observed for the appearance of plaques. Titers were determined by counting viral plaques at 2 weeks.

2.5 Formulation and Quality Control Parameters

The final production of the AdCFTR and Av1CF1 vectors involves: (1) purification of virions away from the intracellular debris of the transfected 293 cells by low speed centrifugation; (2) CsCl step gradients to remove low molecular weight cellular and unincorporated viral protein components; (3) CsCl isopycnic density gradient centrifugation to remove incomplete virions and empty viral capsids (all have lower density than infectious adenovirus); and (4) dialysis to remove low molecular weight components, including the CsCl used for the gradient centrifugation. To maintain stable, intact, infectious recombinant adenovirus during and subsequent to dialysis, the final dialysate includes 10 mM Tris-Cl, pH 7.4, 1 mM Mg Cl_2, and 10% glycerol (to prevent aggregation of virions).

The final stored preparation to be used in the clinical protocol consists of AdCFTR at a titer of 10^{11} to 10^{12} pfu/ml in 10 mM Tris-Cl, pH 7.4, 1 mM $MgCl_2$, and 10% glycerol. The preparation is stored at -70° until use. Prior to in vivo administration, the preparation is thawed and diluted to the required concentration in a final buffer of 10 mM Tris-Cl, pH 7.4, 1 mM $MgCl_2$, and 3.3% glycerol.

Quality control measures to be used are designed to assay the 293 cells, the seed virus used for initial infection, the virus prior to purification and the final purified virus for adventitious agents, structure, and function. All studies will be carried out under the U.S. Good Laboratory Practices (GLP) and Regulations as promulgated in Title 21 CFR Section 5.8.

The 293 cell bank will be assessed for bacteria and fungus, sterility, mycoplasma, karyology, (isozyme and cytogenetic analysis), in vitro evaluation for non-specific virus contaminants by evaluating indicator cells for cytopathic effect, in vivo evaluation for non-specific virus using adult mice, suckling mice, guinea pigs and embryonated hens egg for clinical signs of viral infection, Epstein-Barr virus, cytomegalovirus, bovine virus (including bovine viral diarrhea virus, infectious bovine rhinotracheitis virus, parainfluenza 3 virus, replication competent adenoviruses, and bovine parvovirus), hepatitis B virus, human immunodeficiency virus, porcine parvovirus, adenovirus, and electron microscopy (as a general virus screen).

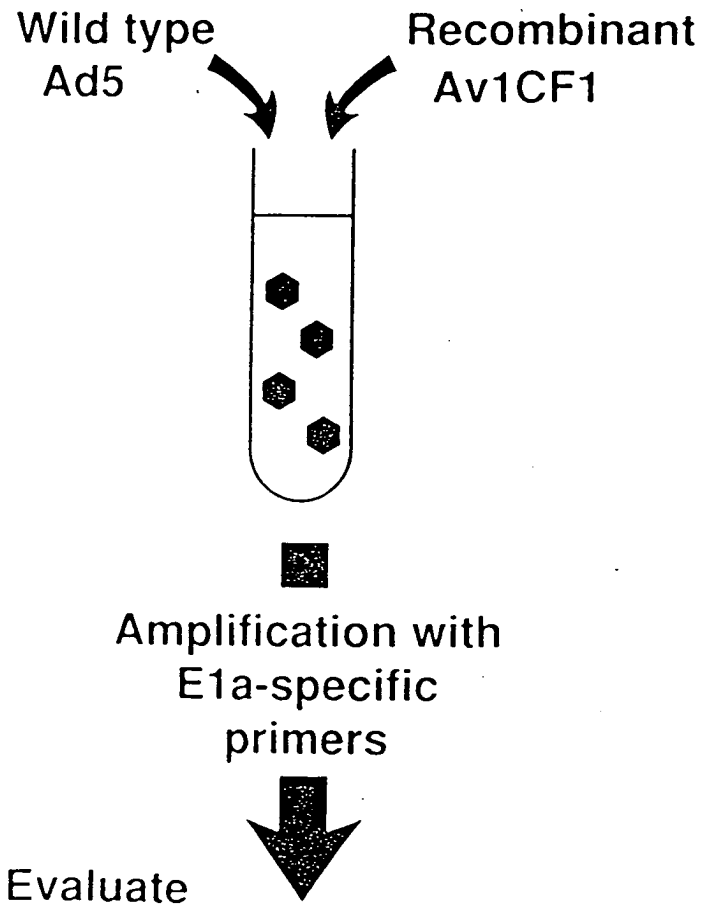
The genome of the AdCFTR seed virus will be evaluated for structural integrity by Southern analysis, DNA sequence of the left end of the vector through the region at the beginning of the large ClaI fragment of Addl324, titer, expression of exogenous CFTR cDNA at the mRNA and protein levels in vitro and in vivo (see sections 3.1, 3.2), expression of viral genes (hexon) at the mRNA and protein levels (see section 4.2), DNA replication in cultured human epithelial cells (HeLa) and in freshly isolated human respiratory epithelial cells (see Section 4.1), for the presence of contaminating adenovirus type 5 Ela sequences (e.g., from contamination in the laboratory), sterility, and adventitious agents.

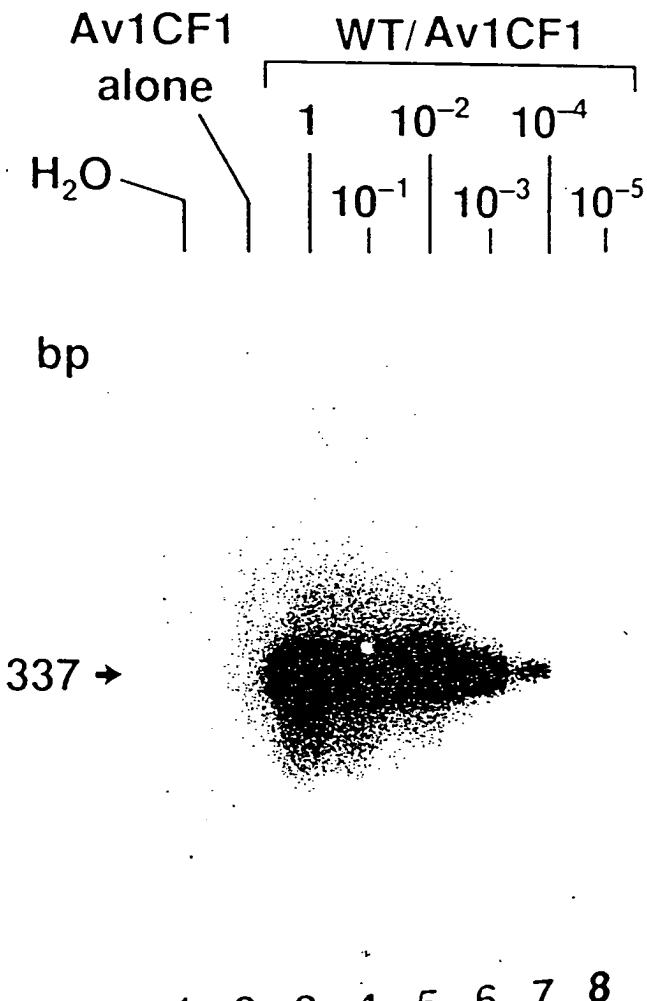
The final purified AdCFTR preparation will be evaluated for sterility, limulus amebocyte lysate (for gram negative bacterial endotoxin) and general safety (inoculation into guinea pigs and mice and observation for overt signs of ill-health or unusual response).

The evaluations listed above will be carried out using standard GLP conditions. The evaluation for the presence of contaminating Ad5 will be carried out by assessing the preparation for the presence of Ad5 Ela sequences. This is accomplished using a PCR-based amplification system with oligonucleotide primers located within the structural portion of the Ela gene (evaluating for the presence of Ad5 sequences 562 to 899 bp; (GenBank accession No. 73260). As a positive control, and to define the sensitivity of the assay, each assay includes a standard curve consisting of mixtures of the recombinant vector and an Ela^+ Ad5 virus. As a control for the presence of DNA, each sample is evaluated by PCR using oligonucleotide primers within the E2b region (Ad2 base numbers 6671 to 7072; Ad5 and the

Ad5-based viruses Ad-dl327 and the recombinant vector are positive with these primers). An example of this assay is shown in Figure 2.5-A for the vector AvlCFl. The assay can detect contaminating intact Ad-dl327 or Ad5 virus in a preparation of AvlCFl at a level of 1 in 10^5 (Figure 2.5-A).

Figure 2.5-A. Assessment of a preparation of an $E1^-$, replication deficient Ad5-based adenovirus for contamination with Ad5. The example shown is for the vector Av1CF1. Mixtures of Ad5 (WT) and Av1CF1 from 1:1 to $1:10^{-5}$ were evaluated by amplification with E1a specific primers followed by southern analysis using a ^{32}P-nested E1a probe. No E1a sequences are present in the H_2O control (lane 1) or Av1CF1 alone (lane 2). E1a sequences are readily detectable in a mixture 1 of Ad5 and Av1CF1 to at least a ratio of $1:10^{-5}$ (lanes 3-8). At the exposure shown the signal at 10^{-5} (lane 8) is not seen; with longer exposures (not shown) a clear signal at 10^{-5} is observed. The expected E1a fragment is 337 bp. See text (section 2.5) for further details.





SECTION 3

EXPERIMENTAL EVIDENCE SUPPORTING THE USE OF A REPLICATION DEFICIENT RECOMBINANT ADENOVIRUS CONTAINING THE HUMAN CFTR cDNA TO TREAT THE RESPIRATORY MANIFESTATIONS OF CYSTIC FIBROSIS

3. Experimental Evidence Supporting the Use of a Replication Deficient Recombinant Adenovirus Containing the Human CFTR cDNA to Treat the Respiratory Manifestations of Cystic Fibrosis.

There is evidence from a variety of viewpoints that a $E1^-$ $E3^-$ replication deficient recombinant adenovirus can transfer the normal human CFTR cDNA to airway epithelial cells and that the CFTR cDNA is expressed and will correct the CF phenotype of epithelial cells from individuals with CF. For additional details, see Appendix 1 (Rosenfeld MA, Yoshimura K, Trapnell BC, Yoneyama K, Rosenthal ER, Dalemans W, Fukayama M, Bargon J, Stier LE, Stratford-Perricaudet L, Perricaudet M, Guggino WB, Pavirani A, Lecocq J-P, Crystal RG. In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. Cell 1992; 68:143-155) and Appendix 2 [Mastrangeli A, Danel C, Rosenfeld MA, Stratford-Perricaudet L, Perricaudet M, Pavirani A, Lecocq J-P, Crystal RG. Diversity of airway epithelial cell targets for in vivo recombinant adenovirus-mediated gene transfer. J Clin Invest (in press)].

3.1 In vitro Evidence

The in vitro evidence demonstrates that a recombinant adenovirus vector containing the CFTR cDNA will transfer the cDNA in a fashion that results in expression of the CFTR gene at the mRNA and protein levels and will complement the abnormal mutations in the endogenous CFTR genes to reestablish the ability of the epithelial cells to secrete Cl^- in response to cAMP. Further, such a vector will survive in the relatively harsh inflammatory milieu of the airway epithelial lining fluid in CF.

3.1.1 Expression of CFTR mRNA

Following AdCFTR or AvlCF1 infection, human CFTR mRNA derived from the adenovirus vector can be detected in a variety of cell lines including human cervical carcinoma (HeLa), cystic fibrosis pancreatic carcinoma cell line (CFPAC-1), human embryonic kidney cells (293), and freshly isolated cystic fibrosis human bronchial cells (data for AdCFTR, see Appendix 1; data for AvlCF1, not shown). Following infection with AdCFTR (but not with controls), in situ hybridization demonstrated human CFTR mRNA in freshly isolated normal human bronchial epithelial cells and cystic fibrosis human bronchial epithelial cells (see Figures 3.1.1-A, 3.1.1-B).

Following infection with AdCFTR, polymerase chain reaction (PCR) evaluation of freshly isolated human bronchial cells derived from individuals with cystic fibrosis demonstrated AdCFTR-directed mRNA transcripts (Figure 3.1.1-C). Further, whereas prior to infection and after infection with a control virus, these cells expressed only mRNA transcripts with the endogenous CFTR transcripts expressing the CF mutations. After AdCFTR infection, normal CFTR transcripts were present, as demonstrated by sequence analysis and by hybridization with specific probes (not shown).

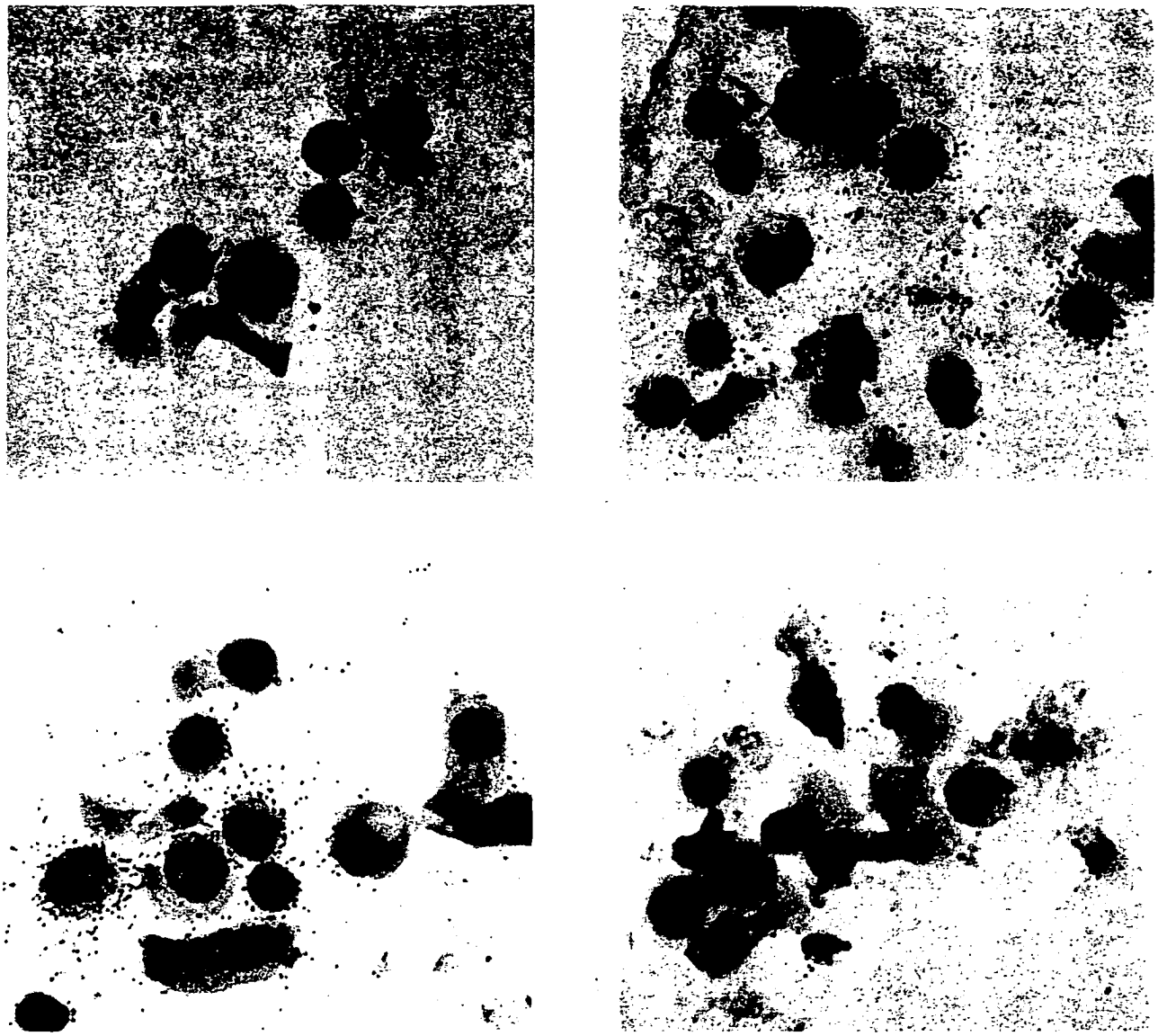


Figure 3.1.1-A. Evaluation of Ad-CFTR-directed human CFTR transcripts in human bronchial cells by in situ hybridization. Human bronchial brushing cells (freshly recovered during bronchoscopy by cytologic brush from normal individuals) were infected with Ad-CFTR for 48 hr (Appendix 1; Rosenfeld et al., 1992). Human CFTR mRNA transcripts were evaluated in cytocentrifuge preparations by in situ hybridization with ^{35}S-labeled antisense (A,B,C,) and sense (D) CFTR RNA probes. A. Uninfected cells (control). B-D. Ad-CFTR infected cells.

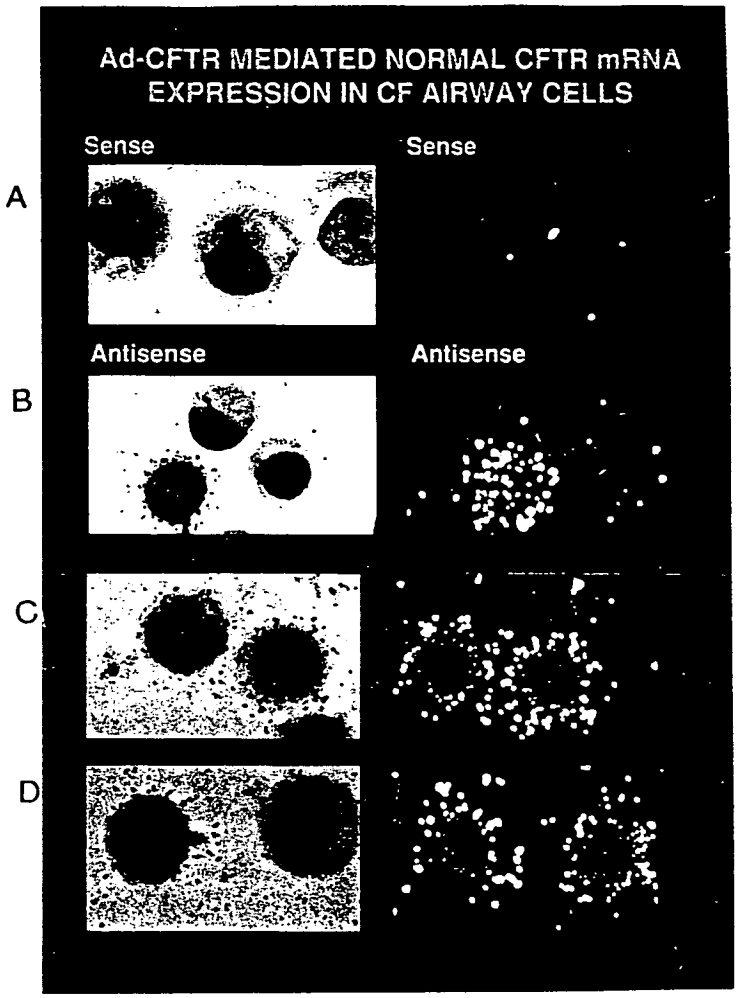
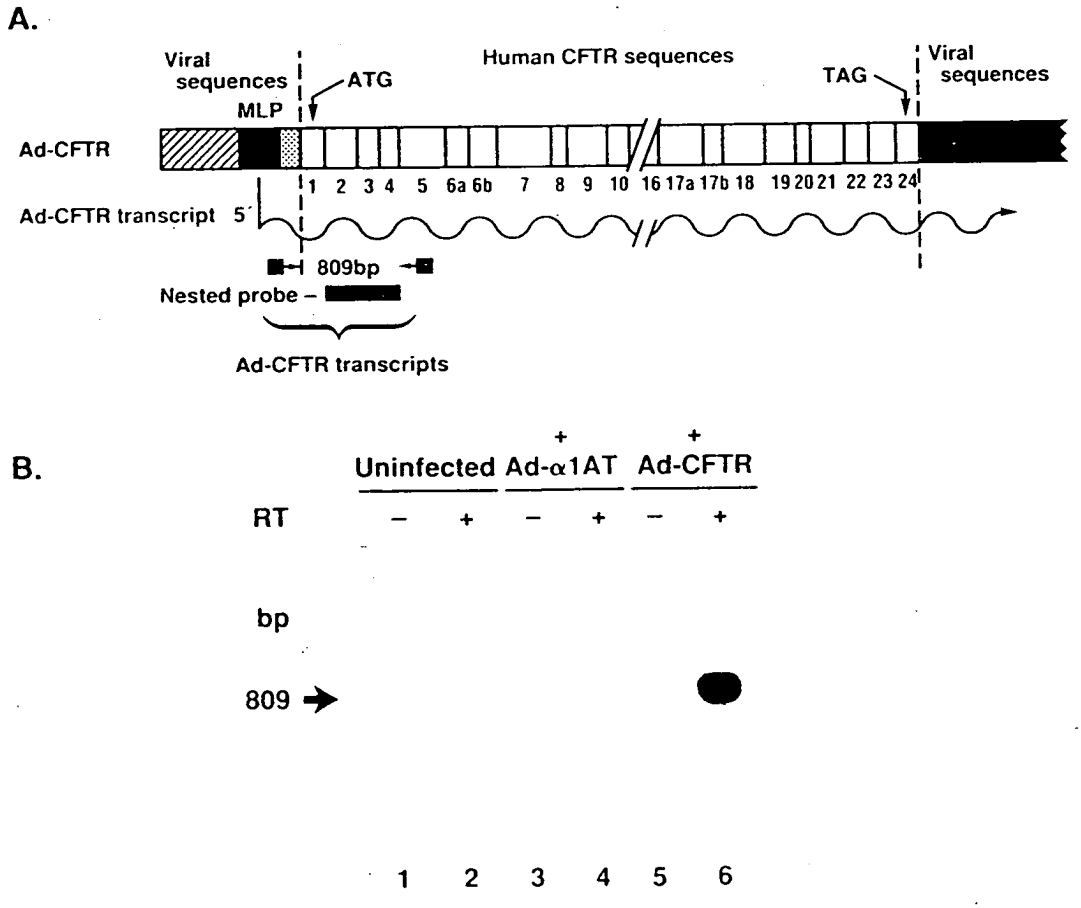


Figure 3.1.1-B. Evaluation of Ad-CFTR-directed human CFTR transcripts in human bronchial cells by in situ hybridization. Human bronchial brushing cells (freshly recovered during bronchoscopy by cytologic brush from individuals with CF) were infected with Ad-CFTR for 24-48 hr (Appendix 1; Rosenfeld et al., 1992). Human CFTR mRNA transcripts were evaluated in cytocentrifuge preparations by in situ hybridization with ^{35}S-labeled sense (A) and antisense (B,C,D) CFTR cRNA probes. Shown are Ad-CFTR infected cells from individuals with CF using direct light microscopy (left) and dark field illumination (right). Uninfected cells had no transcripts evident when evaluated with the antisense probe (not shown).

Figure 3.1.1-C. Evaluation of Ad-CFTR specific CFTR transcripts in human respiratory epithelial cells from an individual with CF following in vitro infection with Ad-CFTR. Human respiratory epithelial cells were obtained during bronchoscopy by cytologic brush, infected with Ad-CFTR and cultured as previously described (Appendix 1; Rosenfeld et al., 1992). RNA was extracted, treated with DNase, converted to cDNA, and amplified with a primer pair consisting of an adenoviral-specific sense primer and a human CFTR-specific antisense primer as previously described (Appendix 1). Each DNase-treated sample was used as a PCR template in parallel without conversion to cDNA to eliminate the possibility of amplifying potentially contaminating viral DNA. PCR products were evaluated by agarose gel electrophoresis followed by Southern hybridization using a nested ^{32}P-labeled human CFTR cDNA probe. Panel A shows the strategy for amplification and hybridization. Note that the location of the 5' primer used for amplification of the cDNA is located in a region of Ad-CFTR that is virus-specific (as compared to CFTR-specific) i.e., the transcripts could only be derived from Ad-CFTR-directed mRNA, not endogenous CFTR mRNA. Panel B shows data from RNA extracted from CF respiratory epithelial cells 24 hr after infection. Lane 1 - uninfected cells, without reverse transcriptase (RT); lane 2 - same as lane 1, with RT; lane 3 - cells after infection with the control virus Ad-αlAT amplified without RT; lane 4 - same as lane 3, with RT; lane 5 - after infection with Ad-CFTR, without RT; and lane 6 - same as lane 5, with RT.



3.1.2 Expression of CFTR Protein

Following infection with AdCFTR (but not in uninfected cells), human CFTR protein can be detected by immunohistochemistry in a variety of cell lines and freshly isolated cells, including 293 cells, HeLa cells, IB3-1 cells, freshly isolated normal human bronchial cells, freshly isolated cystic fibrosis human bronchial epithelial cells, freshly isolated cotton rat tracheal-bronchial cells, and freshly isolated rhesus bronchial cells (see Appendix 1, Figure 3.1.2-A, Figure 3.1.2-B). Using identical methods, human CFTR protein can also be detected following AvlCFl infection of 293 cells, HeLa cells, IB3-1 cells, and freshly isolated normal human bronchial epithelial cells (not shown). In contrast, expression of the endogenous CFTR gene in these cells is low and endogenous human CFTR protein cannot be detected in freshly isolated tissues by this immunohistochemistry assay. This is true for cells from experimental animals and from humans.

Following infection with Ad-CFTR (but not control vectors, or in uninfected cells), human CFTR protein can be immunoprecipitated and subsequently detected after phosphorylation with protein kinase A from a variety of cells including 293, freshly isolated normal human airway epithelial cells, and freshly isolated cotton rat tracheal-bronchial epithelial cells (Figure 3.1.2-C).

Following infection with Ad-CFTR (but not control vectors, or in uninfected cells), de novo synthesized human CFTR protein can be detected by ^{35}S-methionine labeling, immunoprecepitation, sodium dodecyl sulfate polyacrylamide gels, and autoradiography in 293, CFPAC-1 and freshly isolated normal human airway epithelial cells (Figure 3.1.2-D and Appendix 1).

3.1.3 Functional "Complementation" of the Cystic Fibrosis Phenotype

Consistent with the role of the CFTR protein as a cAMP-regulatable Cl^- channel in epithelia, the biologic phenotype of CF epithelia is the inability to secrete Cl^- in response to an increase in intracellular levels of cAMP. This can be corrected by infection of CF epithelial cells with Ad-CFTR or AvlCFl. The assay used to demonstrate this utilizes quantification of $^{36}Cl^-$ secretion in response to increased cAMP (see Appendix 1 for details). An example of the functional consequences of AvlCFl infection demonstrated with the $^{36}Cl^-$ assay in epithelial cells from individuals with CF (CFPAC-1) is shown in Figure 3.1.3-A. In contrast, a control vector had no effect. The vector AdCFTR will convey cAMP stimulatable Cl^- secretion to cells that do not normally have this function, and to pancreatic cells and airway epithelial cells from individuals with cystic fibrosis (Appendix 1, Figure 3.1.3-B).

3.1.4 Can the Vector Survive in the Airway Epithelial Lining Fluid of Individuals with Cystic Fibrosis?

The airway epithelial milieu of individuals with CF is characterized by an intense neutrophil dominated inflammatory process, and with it, inflammatory cell derived mediators including oxidants, proteases and a variety of

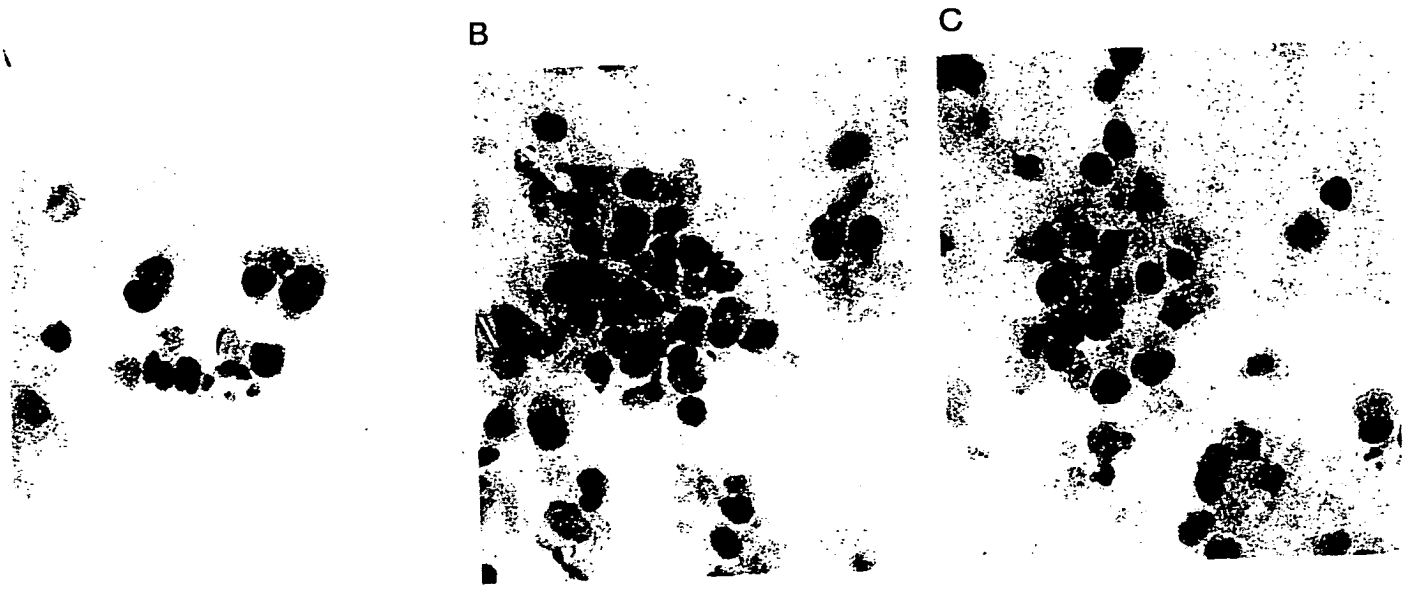
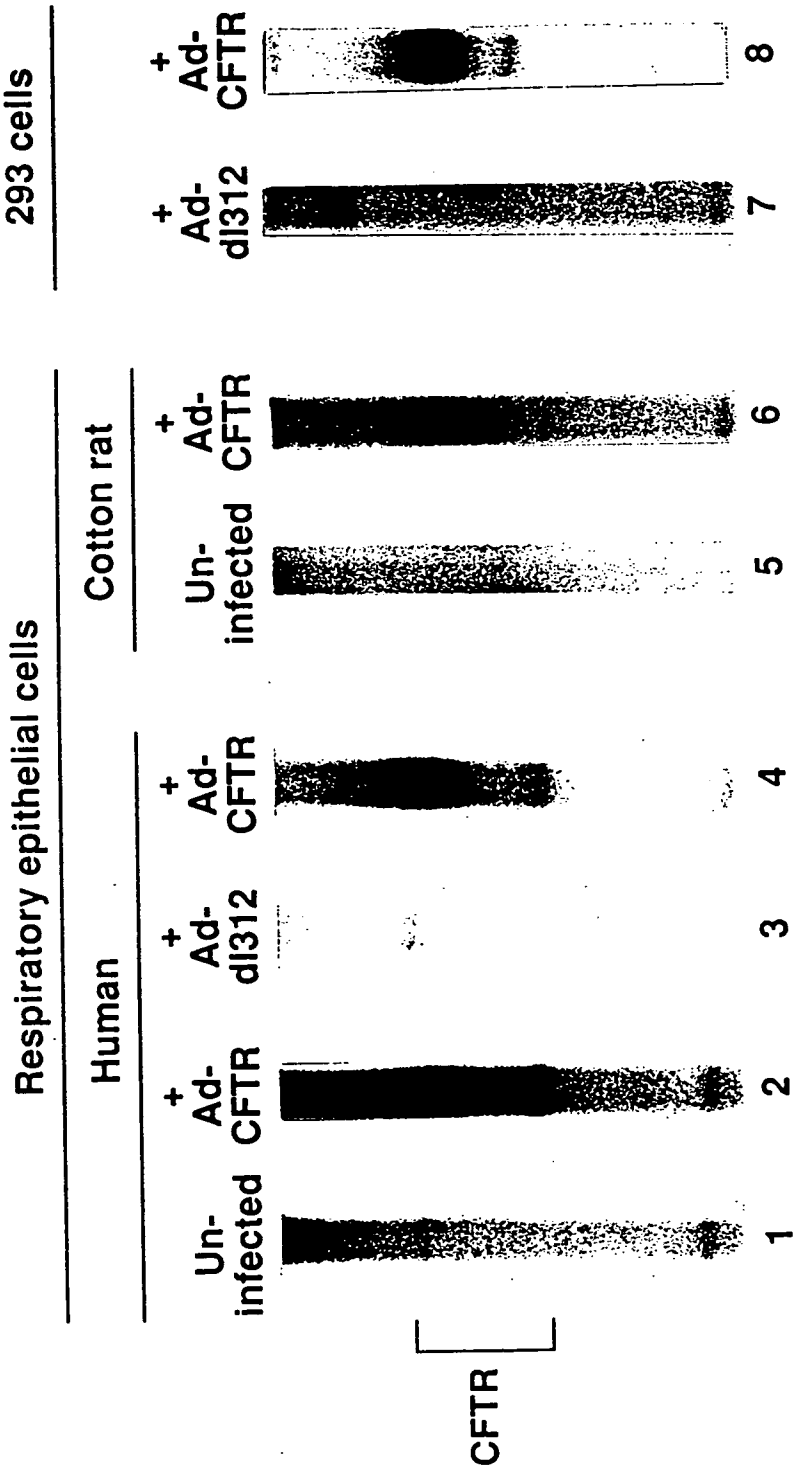


Figure 3.1.2-A. Immunohistochemical detection of CFTR protein in non-human primate (rhesus) respiratory epithelial cells after infection with Ad-CFTR. Rhesus bronchial brushing cells (freshly recovered during bronchoscopy by cytologic brush) were infected in vitro with Ad-CFTR and collected 24 hr later (Appendix 1). Human CFTR protein was evaluated in cytocentrifuge preparations using the alkaline phosphatase anti-alkaline phosphatase method with minor modifications and anti-human CFTR antibody as previously described (Appendix 1). Shown are control uninfected cells (A), cells infected with Ad-CFTR (B) and as a control, cells infected with Ad-CFTR evaluated with an irrelevant primary antibody (C). Immunoreactivity to human CFTR is indicated by a red color; cell nuclei appear blue as a result of hematoxylin counterstaining.

Figure 3.1.2-B. Immunohistochemical detection of CFTR protein in human respiratory epithelial cells after infection with Ad-CFTR. Human bronchial brushing cells (freshly recovered during bronchoscopy by cytologic brush from individuals with CF) were infected in vitro with Ad-CFTR and evaluated at various time points (Appendix 1; Rosenfeld et al., 1992). Human CFTR protein was evaluated in cytocentrifuge preparations using the alkaline phosphatase anti-alkaline phosphatase method with minor modifications and anti-human CFTR antibody as previously described (Appendix 1). Shown are control uninfected cells cultured for 30 hours (A) and cells infected with Ad-CFTR for 30 hours (B). Panel C shows four individual ciliated cells infected with Ad-CFTR for 48 hours; in the lower right corner of panel C, as a control, the primary antibody was eliminated. Immunoreactivity to human CFTR is indicated by a red color; cell nuclei appear blue as a result of hematoxylin counterstaining.

Figure 3.1.2-C. Detection of human, phosphorylated CFTR protein in respiratory epithelial cells after infection with Ad-CFTR. Human bronchial brushing cells (freshly recovered during bronchoscopy by cytologic brush from normal individuals) and cotton rat tracheal-bronchial brushing cells (recovered by cytologic brush from naive cotton rats) were infected in vitro with Ad-CFTR or the control Ela deletion mutant virus Ad-dl312 and collected 48 hr later (Appendix 1). As a positive control for expression of CFTR following Ad-CFTR infection, 293 cells were infected with Ad-CFTR and collected 16 hr later. CFTR protein was evaluated by immunoprecipitation with a mouse monoclonal antibody against human CFTR followed by phosphorylation using protein kinase and [γ-^{32}P]ATP (Cheng et al., 1990; Bargon et al., 1992). Human bronchial brushing cells from 2 normal individuals (individual #1, lanes 1,2; individual #2, lanes 3,4) cotton rat tracheal-bronchial brushings cells (lanes 5,6) and 293 cells (lanes 7,8) were evaluated. Shown are control uninfected cells (lanes 1,5), cells infected with Ad-dl312 (lane 3,7), and cells infected with Ad-CFTR (lanes 2,4,6,8). The expected size range for CFTR protein is indicated.

Figure 3.1.2-C


Respiratory epithelial cells
Human
Cotton rat
293 cells
Un-infected
+ Ad-CFTR
+ Ad-dl312
Un-infected
+ Ad-CFTR
+ Ad-dl312
+ Ad-CFTR
1
2
3
4
5
6
7
8
CFTR

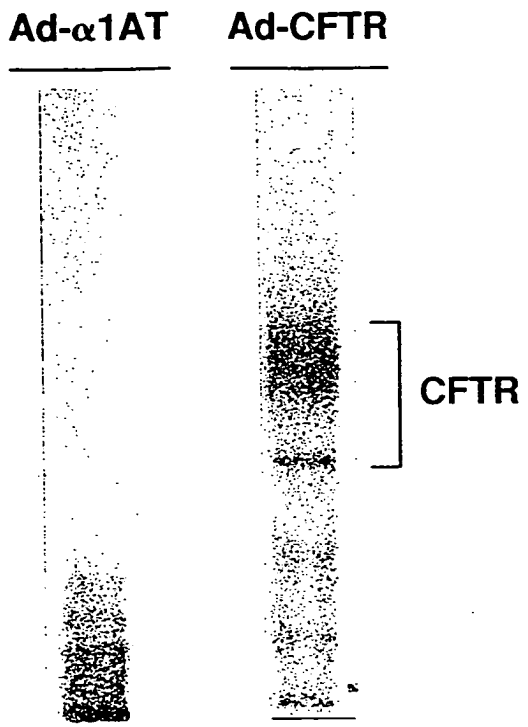


Figure 3.1.2-D. Detection of de novo synthesized CFTR protein in respiratory epithelial cells after infection with Ad-CFTR. Human bronchial brushing cells (freshly recovered during bronchoscopy by cytologic brush from normal individuals) were infected in vitro with Ad-CFTR or the control virus Ad-αlAT (Appendix 1), simultaneously labelled with [^{35}S]methionine and evaluated 48 hr later. Cell lysate was prepared and evaluated for [^{35}S]methionine labelled CFTR by immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography as previously described. Shown are cells infected with the control adenovirus vector (see Rosenfeld et al., 1991) Ad-αlAT (lane 1) and Ad-CFTR (lane 2). The size range for CFTR is indicated.

Figure 3.1.3-A. In vitro evaluation of the function of human CFTR protein directed by AvlCFl. The functional ability of AvlCFl to modulate forskolin-stimulated Cl^- permeability was evaluated in CFPAC-1 cells [pancreatic adenocarcinoma cells from an individual homozygous for the common ΔF508 mutation (Rommen et al., 1989; Riordan et al., 1989; Kerem et al., 1989; Collins, 1992)]. CFPAC-1 cells were trypsinized, counted, seeded (3 cm plates [$5x10^5$ cells/plate]) and infected in suspension at 200 pfu/cell with AvlCFl or AvlNull (a control vector identical to AvlCFl except that the CFTR cDNA is deleted). Cl^- efflux was evaluated 48 hr after infection at rest (basal) and after stimulation (forskolin) as previously described (Appendix 1; Trapnell et al., 1991b) in uninfected CFPAC-1 cells (A), cells infected with AvlNulll (B) and cells infected with AvlCFl (C).

Figure 3.1.3-A

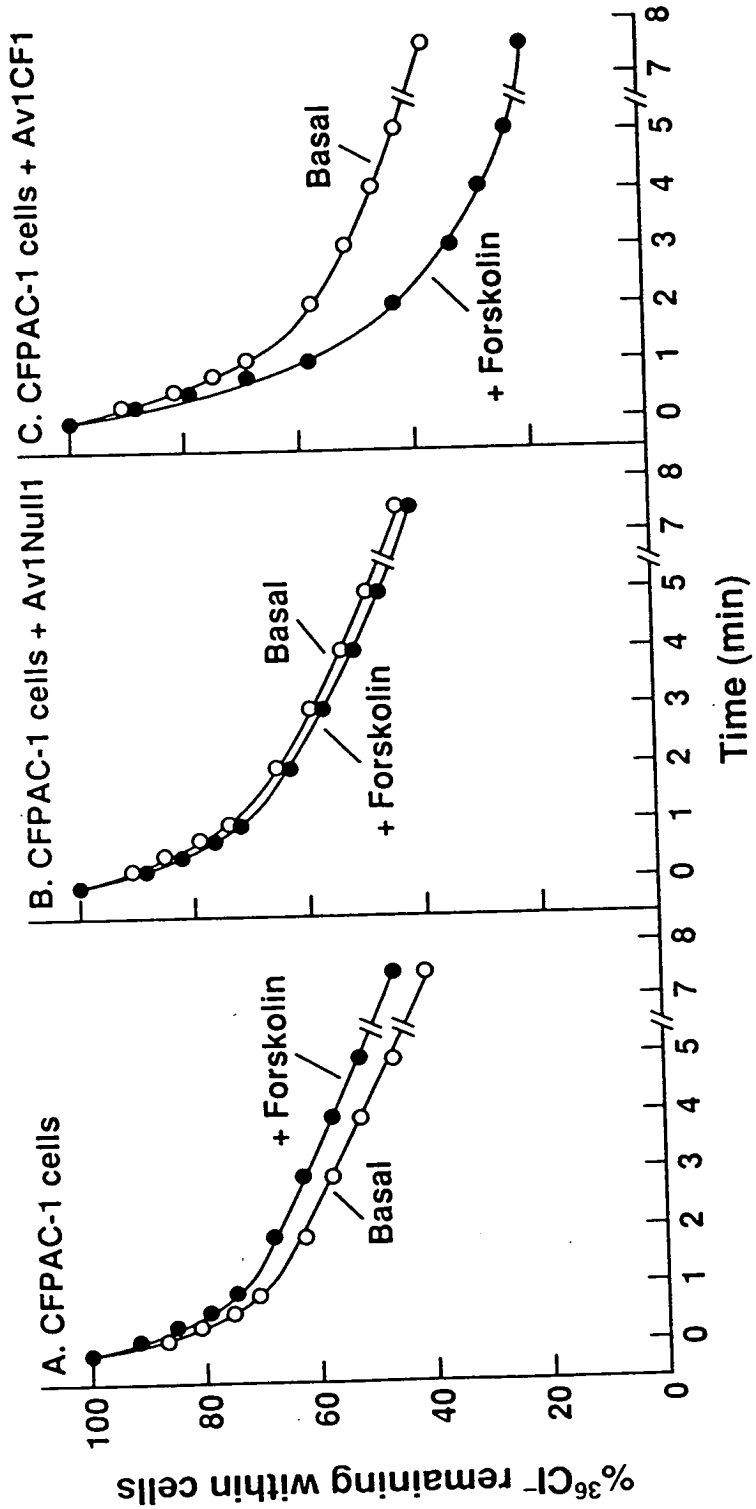
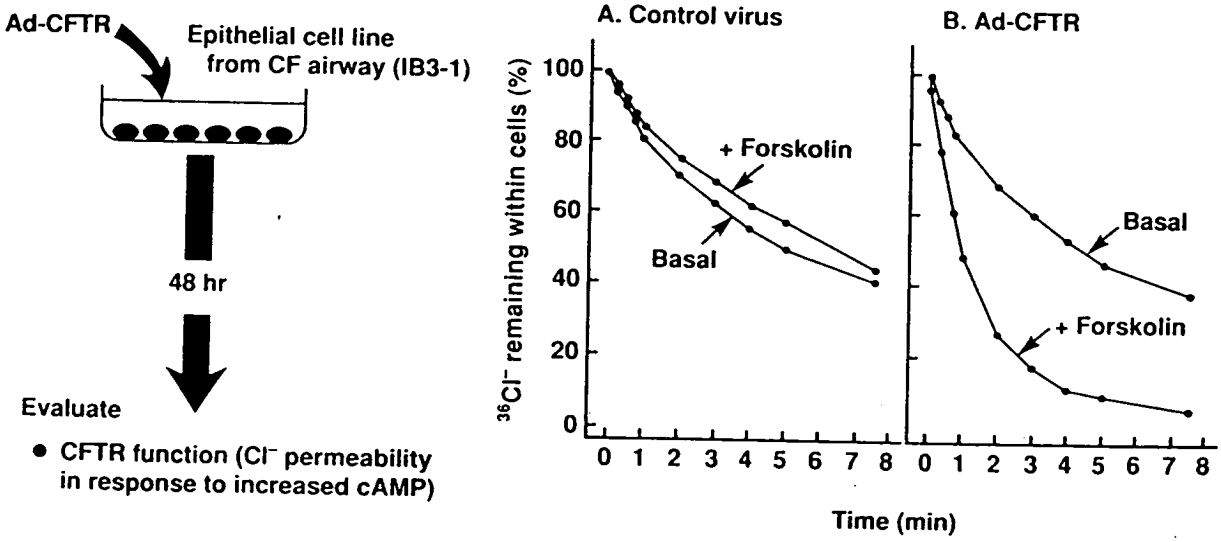

A. CFPAC-1 cells
+ Forskolin
Basal
B. CFPAC-1 cells + Av1Null1
Basal
+ Forskolin
C. CFPAC-1 cells + Av1CF1
Basal
+ Forskolin
Time (min)
%36Cl- remaining within cells
100
80
60
40
20
0
0 1 2 3 4 5 7 8

Figure 3.1.3-B. In vitro evaluation of the function of human CFTR protein directed by AdCFTR. The functional ability of AdCFTR to modulate forskolin-stimulated Cl^- permeability was evaluated in IB3-1 cells [epithelial cell line derived from an individual with cystic fibrosis (Zeitlin et al. 1991)] IB3-1 cells were trypsinized, counted, seeded (3 cm plates [$5x10^5$ cells/plate]) and infected in suspension at 200 pfu/cell with AdCFTR or a control vector (AvlNull, an $E1^-E3^-$ repliction deficient adenovirus identical to AvlCFl, but with no CFTR cDNA. Cl^- efflux was evaluated 48 hr after infection at rest (basal) and after stimulation (forskolin) as previously described (Appendix 1; Trapnell et al., 1991b) in (A) cells infected with AvlNull and (B) IB3-1 cells infected with AdCFTR.



cytokines (Boat et al., 1989; McElvaney et al., 1991; McElvaney et al., 1992; Suter et al., 1988; Suter et al., 1989a; Welsh and Fick, 1987). To insure that a replication deficient recombinant adenovirus can survive in this hostile environment, AdRSV.βgal, a recombinant vector containing the E. coli lacZ gene coding for β-galactosidase (see Appendix 2 for details) was incubated at 37° in respiratory epithelial lining fluid from individuals with CF for varying times and the fluid (with the vector) then placed on HeLa cells. Evaluation of the cells after 24 hr demonstrated the vector had infected the cells and the β-galactosidase gene was expressed (Figure 3.1.4-A).

3.2 In Vivo Evidence

The evidence that a recombinant vector containing the CFTR cDNA will transfer the cDNA to the airway epithelium in vivo comes from studies in cotton rats [a rodent with susceptibility to human adenovirus infection similar to that of humans (Ginsberg et al., 1989)] and in non-human primates (rhesus).

3.2.1 Expression In Vivo in Cotton Rats

Studies with cotton rats have demonstrated that the intratracheal administration of Ad-CFTR results in expression of the human CFTR cDNA as evidenced by: (a) PCR analysis showing human CFTR mRNA expression in the lung for at least 6 weeks; (b) in situ hybridization analysis demonstrating human CFTR mRNA transcripts in airway epithelial cells; (c) quantitative northern analysis showing that lung levels of human CFTR mRNA transcripts at 6 weeks are approximately 42% of that at 2 days after instillation; and (d) immunohistochemical evidence of human CFTR protein in airway epithelial cells. Details of these studies can be found in Appendix 1.

In regards to the relative infectivity and resulting expression of the exogenous gene in various airway epithelial cells in vivo, studies were carried out in cotton rats receiving intratracheal AdRSV.β gal, the recombinant vector containing the β-galactosidase gene. The results demonstrate that all major categories in the airway epithelium of large and small airways are equally infectable, including ciliated, basal, secretory and undifferentiated columnar cells (see Appendix 2 for details).

3.2.2 Expression In Vivo in Non-Human Primates

Intratracheal administration of the recombinant adenovirus vector containing the lacZ gene to rhesus demonstrated expression of β-galactosidase protein in airway epithelial cells at 3 days (Figure 3.2.2-A) and 7 days (not shown) after administration.

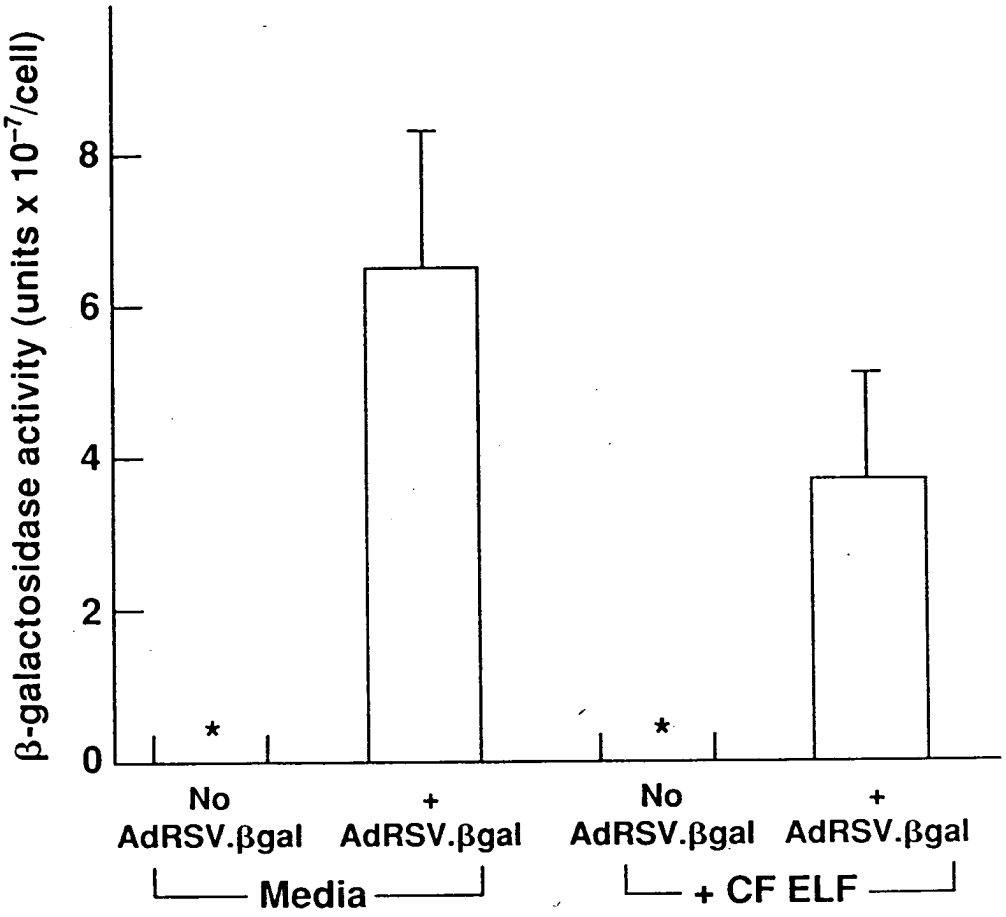
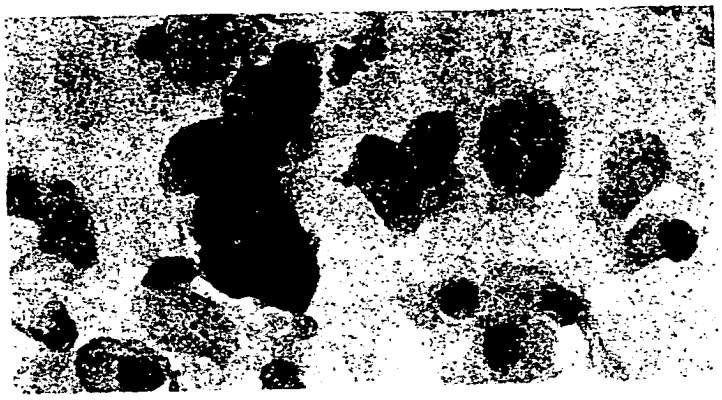


Figure 3.1.4-A. Effect of epithelial lining fluid of individuals with cystic fibrosis on the in vitro expression of a recombinant, replication deficient adenovirus vector. AdRSV.βgal (10^7 pfu) was incubated (30 min, 37^0) with media or epithelial lining fluid (ELF) from an individual with cystic fibrosis. The various fluids were then added to HeLa cells (10^6 cells, 10 pfu/cell) and the incubation continued for 24 hrs at 37^0. β-galactosidase activity was evaluated in cell lysates by spectrophotometry (420 nm); see Appendix 2 for details regarding the vector and assay methods).

A



B

Figure 3.2.2-A. Histochemical detection of E.Coli ß-galactosidase in rhesus monkey respiratory epithelial cells after in vivo infection with AdRSV.ßgal. Rhesus monkey tracheal epithelial cells were infected in vivo with $2x10^9$ pfu/kg of an adenovirus vector derived from type 5 adenovirus and containing the E. coli lacZ gene which codes for the ß-galactosidase protein (see Appendix 2). After 3 days, epithelial cells were recovered from the trachea by bronchoscopic visualization and removal using a cytology sampling brush. ß-galactosidase was evaluated in cytocentrifuge preparations using X-gal as a substrate. Samples were stained with hematoxylin and eosin. Shown are: (A) epithelial cells obtain from a control animal administered vehicle; and (B) epithelial cells obtained from an animal administered AdRSV.ßgal. The presence of ß-galactosidase is indicated by a dark blue nuclear staining.

SECTION 4

SAFETY CONSIDERATIONS

4. Safety Considerations

There are a variety of safety issues relevant to the use of a replication deficient recombinant adenovirus to treat the respiratory manifestations of CF, including: will the vector replicate in human epithelial cells? Does the vector express viral genes in addition to the CFTR cDNA? Is respiratory infection with the vector associated with transfer of the exogenous gene to the germ line? Is respiratory administration of the vector associated with damage to the lung? Are anti-vector antibodies elicited by respiratory administration of the vector? If so, does reinfection with another adenovirus pose a risk? Is the vector shed following respiratory administration of the vector? Are there risks of having some of E3 deleted from the vector? If recombination or complementation of the vector occurs *in vivo*, does this pose a risk to the patient and/or environment? Is there a risk of malignancy associated with the use of this vector? Does the genome of the vector integrate into the genome of the target cells? Does the expression of the newly transferred CFTR cDNA need to be regulated?

The following addresses these questions point by point, and details *in vitro* and *in vivo* evidence that leads to the conclusion that the potential benefits of the use of this vector in the proposed clinical protocol significantly outweigh the potential risks accompanying the use of this vector.

4.1 Will the Vector Replicate in Human Epithelial Cells?

With the deletion of map units 1.26 to 9.24 (455 to 3327 bp of the left hand end), Ad-CFTR and AvlCF1 have been designed to eliminate the regions of the adenovirus genome that control replication of adenovirus DNA and thus replication of the vector. To evaluate whether an $E1^-E3^-$ recombinant adenovirus vector replicates in human epithelial cells, human cervical carcinoma cells (HeLa), a human epithelial cell line known to be permissive for wild type adenovirus growth (Cinatl et al., 1992), were infected with AvlCF1 (60 pfu/cell) and the cells evaluated over time by assessing cell lysates for the presence of infectious adenovirus (or intact vector DNA) by adding the lysate to 293 cells and determining the resulting titer of adenovirus. No increase in the amounts of infectious virions was observed (Figure 4.1-A). In contrast, the control virus (Ad-*dl*327, an $E1^+E3^-$ Ad5-based virus) increased over time.

To determine whether vector DNA replication occurs, HeLa cells were infected with AvlCF1 (30 to 1000 pfu/cell), the cells incubated with $^{32}PO_4^{=}$, viral DNA extracted (by a modified Hirt procedure, Hirt, 1967), the DNA cut with EcoR1, and gel electrophoresis and autoradiography were carried out. Whereas Ad-*dl*327 infection was clearly associated with viral DNA replication at all multiplicity of infection (MOI), AvlCF1 replication in HeLa cells was MOI dependent (Figure 4.1-B). At low MOI (MOI of 30 or less) no DNA replication was observed but at high MOI (MOI of 100 or more), HeLa did support AvlCF1 DNA replication. This result is consistent with the observation that infection of HeLa at 60 pfu/cell was not associated with an increase in the amount of infectious virions (Figure 4.1-A). It is also consistent with evidence that at high MOI, HeLa will support replication of $E1a^-$ adenovirus

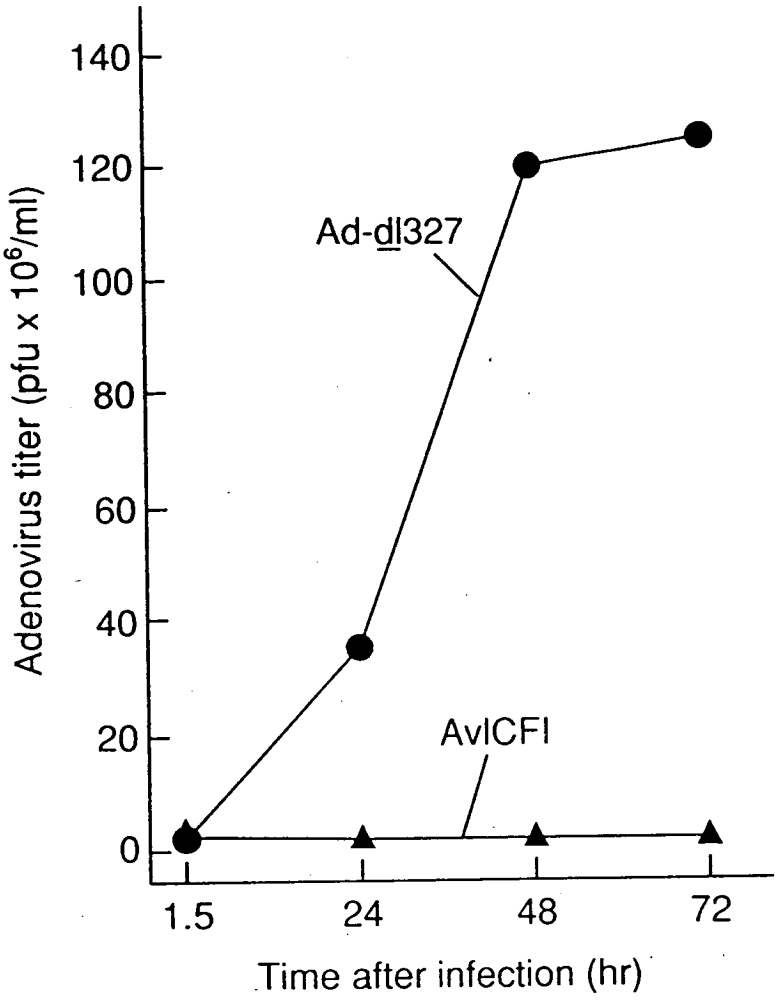
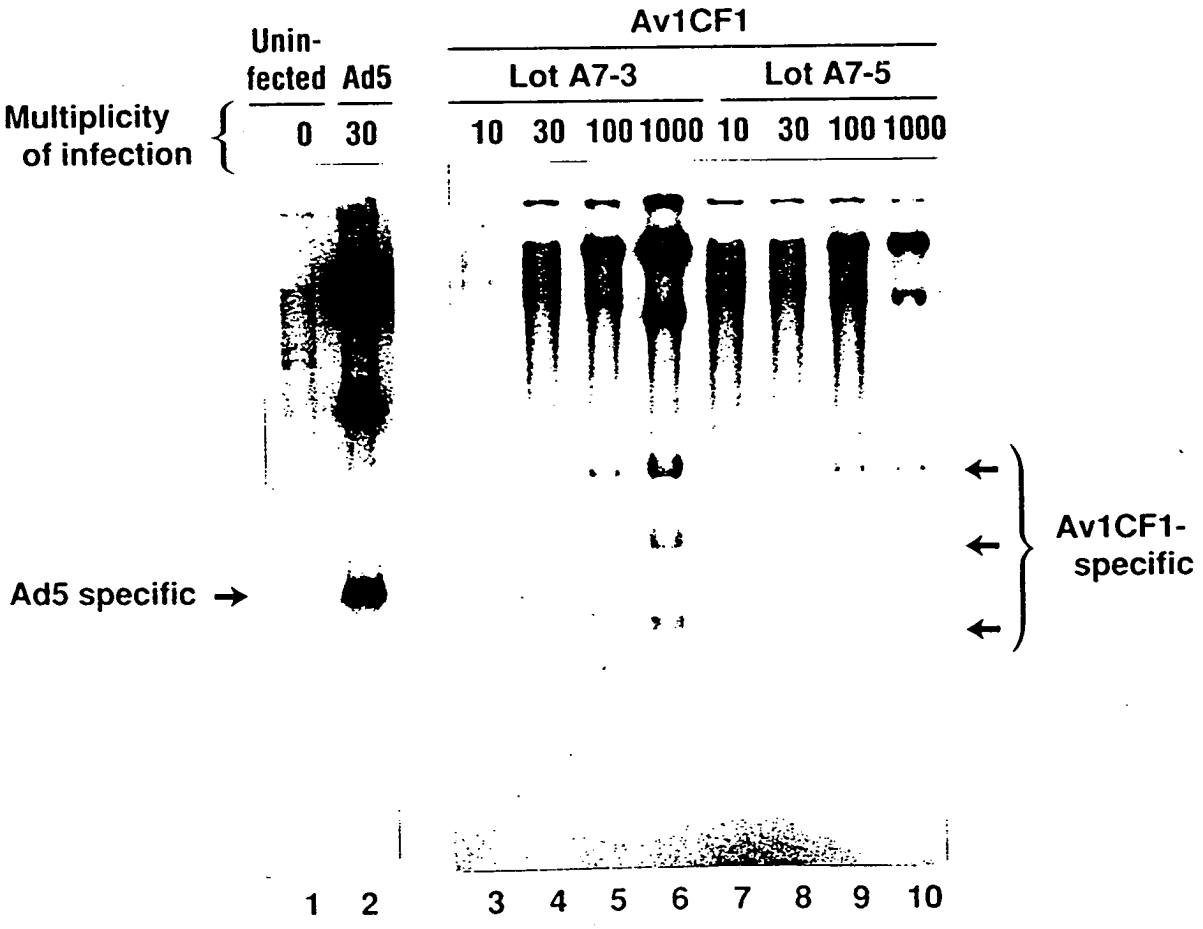


Figure 4.1-A. Evaluation of the replication potential of AvlCFl in HeLa cells by measurement of production of infectious virus. HeLa cells were grown to 80% confluency in Dulbecco's modified Eagle medium, 10% fetal calf serum, 1% glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and infected with AvlCFl or Ad-dl327 at a multiplicity of infection (MOI) of 60 plaque forming units (pfu) per cell. After 90 minutes ("0" time), 24 hr, 48 hr, or 72 hr, cells and media were collected and all cells were recovered and freeze-thawed 5 times to release intracellular adenovirus. Serial dilutions of the freeze-thaw lysate were prepared and used to infect 293 cells grown to 95% confluency. Ninety minutes after infection, the media was replaced with soft agarose overlay media (Appendix 1; Green et al., 1967) and the cells were incubated at 37°C, 5% CO_2 in a humidified atmosphere. After two weeks, the plates were evaluated for the presence of adenoviral plaques. The titer of adenovirus was calculated from the number of plaques at a given dilution and the dilution factor.

Figure 4.1-B. Evaluation of the replication potential of AvlCF1 DNA in HeLa cells by ^{32}P-labelling of DNA after infection of HeLa cells by AvlCF1. HeLa cells were grown to 80% confluency in Dulbecco's modified Eagle medium (DMEM), 10% fetal calf serum, 1% glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and infected with AvlCF1 at a multiplicity of infection (MOI) of 10 to 1000 plaque forming units (pfu) per cell (Appendix 1). After 20 hr, the media was replaced with phosphate-free DMEM containing $^{32}PO_4^=$ (Berkner and Sharp, 1983) as well as supplements described above. Following a 20 hr labeling period, cells were lysed and viral DNA was prepared by the Hirt extraction method (Hirt, 1967). The DNA was purified by proteinase K treatment and phenol extraction, cleaved with EcoRI and evaluated by agarose gel electrophoresis. Lane 1- uninfected control (note the doublet of mitochondrial DNA). Lane 2- positive control infection with Ad5. Lane 3- AvlCF1 lot A7-3, MOI 10; Lane 4-6, same as lane 1 but at MOI of 30, 100, 1000 respectively. Lanes 7-10, same as lanes 3-6, but with AvlCF1 lot A7-5. No AvlCF1 DNA replication was observed at MOI of 10 or 30, but at higher MOI (100, 1000), HeLa will support AvlCF1 replication.



mutants (Shenk et al., 1980).

To evaluate whether DNA replication occurs in human airway epithelium following infection with an $E1^-E3^-$ recombinant adenovirus vector, bronchial epithelial cells were recovered by brushing via a bronchoscope from normals. Interestingly, and different from that observed with HeLa, no Av1CF1 DNA replication was observed as a function of time or multiplicity of infection (up to 1000 pfu/cell) using the $^{32}PO_4^=$ DNA labeling technique described above. In contrast, infection with the control Ad5 virus did result in DNA replication (Figure 4.1-C).

As with Av1CF1, infection of human airway epithelium with AdCFTR (MOI up to 1000 pfu/cell) and evaluation using $^{32}PO_4^=$ DNA labeling showed no DNA replication (Figure 4.1-D). In contrast, infection with the control Ad5 virus did result in DNA replication.

The conclusion from these studies is that despite the observations of DNA replication in HeLa at high MOI, no viral DNA replication is detected with $E1^-E3^-$ adenovirus vectors of the design of Av1CF1 and AdCFTR in freshly isolated human airway epithelial cells even at very high MOIs (1000 pfu/cell). To ensure that AdCFTR will not replicate in the respiratory epithelial cells of the individuals to be treated with AdCFTR, the replication potential of AdCFTR will be assessed prior to therapy, with inclusion of the individual only if AdCFTR does not replicate in autologous respiratory epithelial cells (see Section 5.4). To decrease the possibility that complementation may occur, individuals will be screened for the presence of adenovirus in the respiratory tract (see section 4.9) and measures will be taken to prevent adenovirus infection by isolation before and after therapy (see section 5.6).

4.2 Does the Vector Express Viral Genes in Addition to the CFTR cDNA?

From the design of the vector, and from what is known about transcriptional control of the adenovirus, it is reasonable to expect that some viral genes may be expressed following AdCFTR infection of human epithelial cells but at a low level. To evaluate the potential for viral gene expression HeLa cells were infected (100 pfu/cell) with Av1CF1 or wild type virus Ad5 and hexon mRNA and protein (polypeptide II, L3 transcription unit) were examined as a function of time (Figure 4.2-A, 4.2-B). Hexon was chosen because it is the major capsid protein representing approximately half the total protein content of the virus (see section 2.2.1). As expected, HeLa cells infected with Ad5 rapidly produced large amounts of hexon protein. In contrast, hexon mRNA was barely detectable in Av1CF1 infected cells and only then if the autoradiograms were markedly over-exposed (50-fold; see Figure 4.2-A). Consistent with the hexon mRNA data, immunoprecipitation with an anti-hexon antibody of ^{35}S-methionine labeled HeLa cells showed a large amount of <u>de novo</u> hexon biosynthesis soon after Ad5 infection. In contrast, parallel studies of HeLa infected with Av1CF1 showed detectable, but far less hexon biosynthesis with a much delayed kinetics (Figure 4.2-B). The same results were observed without immunoprecipitation i.e., ^{35}S-methionine labeled HeLa cells infected with Ad5 for 36 hr previously (labeling in the last 12 hr) showed a large amount of <u>de novo</u> hexon biosynthe-

Figure 4.1-C. Evaluation of the replication potential of AvlCF1 DNA in freshly isolated normal human bronchial epithelial (HBE) cells. The replication potential of AvlCF1 in HBE cells was evaluated by $^{32}PO_4^=$ labeling of nascent DNA after infection of HBE cells by the recombinant adenovirus AvlCF1. Human bronchial epithelial cells were obtained at bronchoscopy using a standard cytology brush (Trapnell et al., 1991a) and were cultured in suspension in DMEM, 10% fetal bovine serum, 1% glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and infected with AvlCF1 at a MOI of 100 pfu/cell for 48 or 72 hr, or at various MOIs (10, 100, 500, 1000 pfu/cell) for 48 hrs. The media was replaced with phosphate-free DMEM containing inorganic $^{32}PO_4^=$ and the supplements described above. Following a 20 hr labeling period, cells were lysed and viral DNA was prepared by the Hirt extraction method (Hirt, 1967). DNA was purified by proteinase K treatment and phenol extraction, cleaved with EcoRI and evaluated by agarose gel electrophoresis. No labeled viral DNA is seen in control, uninfected cells (lane 1). As expected, infection by Ad5 (MOI = 100) resulted in ^{32}P-labeled viral DNA restriction fragments of the sizes expected for Ad5 after 48 (lane 2) and 72 (lane 3) hr. In contrast, infection by AvlCF1 (MOI = 100) did not generate labeled DNA fragments at 48 hr (lane 4) or 72 hr (lane 5) indicating a lack of replication of AvlCF1 DNA in HBE cells. As a positive control for the function of AvlCF1, AvlCF1 infected cells showed CFTR protein detected by immunohistochemistry (not shown). As a positive control for the assay, infection of HBE cells with Ad5 MOIs ranging from 10 to 1000 showed ^{32}P-labeled DNA restriction fragments of Ad5 (lanes 7-10) indicating replication of Ad5 in HBE cells. In contrast, infection of HBE cells with AvlCF1 at MOIs ranging from 10 to 1000 showed no labeled DNA fragments (lanes 11-14) indicating no replication of AvlCF1 DNA in HBE cells.

Figure 4.1-C

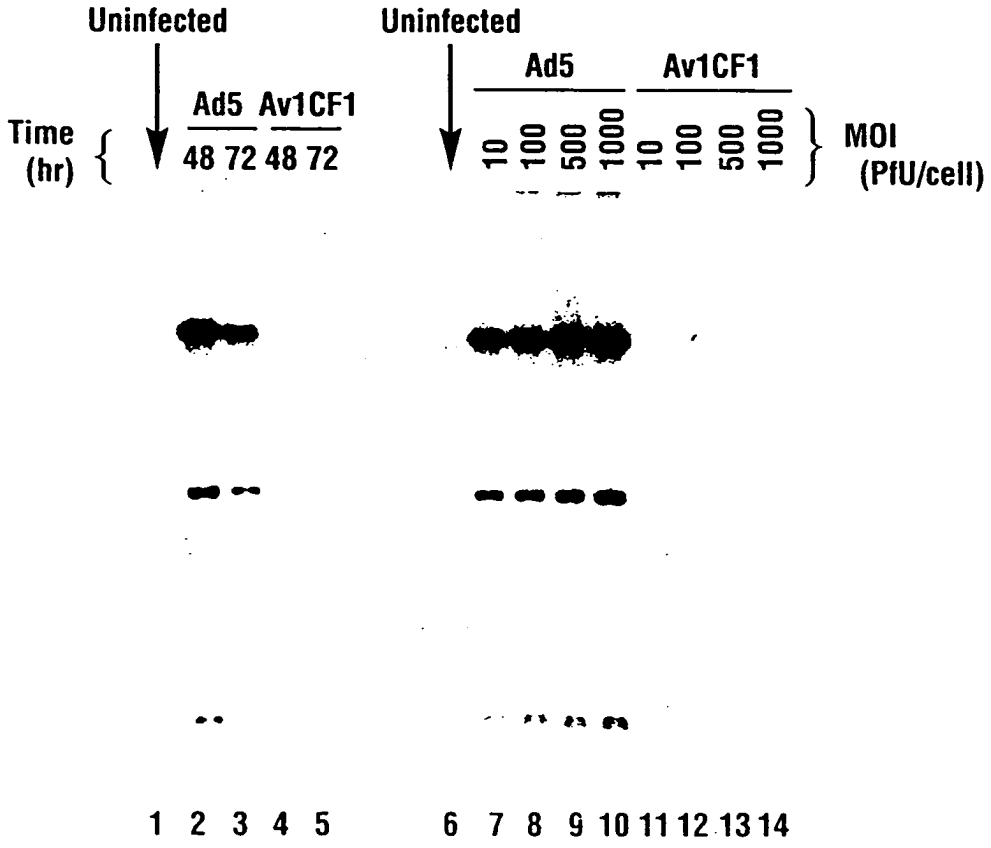

Uninfected
Uninfected
Ad5
Av1CF1
Ad5 Av1CF1
Time (hr)
48 72 48 72
10 100 500 1000 10 100 500 1000
MOI (PfU/cell)
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 4.1-D. Evaluation of the replication potential of AdCFTR DNA in freshly isolated normal human bronchial epithelial (HBE) cells. The replication potential of AdCFTR in HBE cells was evaluated by $^{32}PO_4^{=}$ labeling of nascent DNA after infection of HBE cells by the recombinant adenovirus AdCFTR. Human bronchial epithelial cells were obtained at bronchoscopy using a standard cytology brush (Trapnell et al., 1991a) and were cultured in suspension in DMEM, 10% fetal bovine serum, 1% glutamine, 100 u/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml Fungizone and infected with AdCFTR at a MOI of 100 pfu/cell for 48 or 72 hr, or at various MOIs (10, 100, 500, 1000 pfu/cell) for 48 hrs. The media was replaced with phosphate-free DMEM containing inorganic $^{32}PO_4^{=}$ and the supplements described above. Following a 20 hr labeling period, cells were lysed and viral DNA was prepared by the Hirt extraction method (Hirt, 1967). DNA was purified by proteinase K treatment and phenol extraction, cleaved with EcoRI and evaluated by agarose gel electrophoresis. As a positive control for viral DNA replication, 293 cells were infected with Ad5 or AdCFTR (10 pfu/cell), labeled simultaneously with $^{32}PO_4^{=}$ for 16 hr, and then processed as above. No viral DNA is seen in control uninfected 293 cells (lane 1). As expected, infection with Ad5 resulted in three ^{32}P-labeled viral DNA restriction fragments of the predicted sizes for Ad5 (lane 2; 27 kb, 5.6 kb, and 2.7 kb). Infection by AdCFTR resulted in four ^{32}P-labeled viral DNA restriction fragments of the predicted sizes for AdCFTR (lane 3; 25 kb, 6.4 kb, 3.3 kb and 1.5 kb). The doublet of mitochondrial DNA is indicated by "M". No labeled viral DNA is seen in control, uninfected HBE cells (lane 4). As expected, infection by Ad5 (MOI = 100) resulted in ^{32}P-labeled viral DNA restriction fragments of the sizes expected for Ad5 after 48 (lane 5) and 72 (lane 6) hr. In contrast, infection by AdCFTR (MOI = 100) did not generate labeled DNA fragments at 48 hr (lane 7) or 72 hr (lane 8) indicating a lack of replication of AdCFTR DNA in HBE cells. As a positive control for the assay in HBE cells, infection of HBE cells with Ad5 at MOIs ranging from 10 to 1000 showed ^{32}P-labeled DNA restriction fragments of Ad5 (lanes 9-13) indicating replication of Ad5 in HBE cells. In contrast, infection of HBE cells with AdCFTR at MOIs ranging from 10 to 1000 showed no labeled DNA fragments (lanes 14-17) indicating no replication of AdCFTR DNA in HBE cells.

Figure 4.1-D

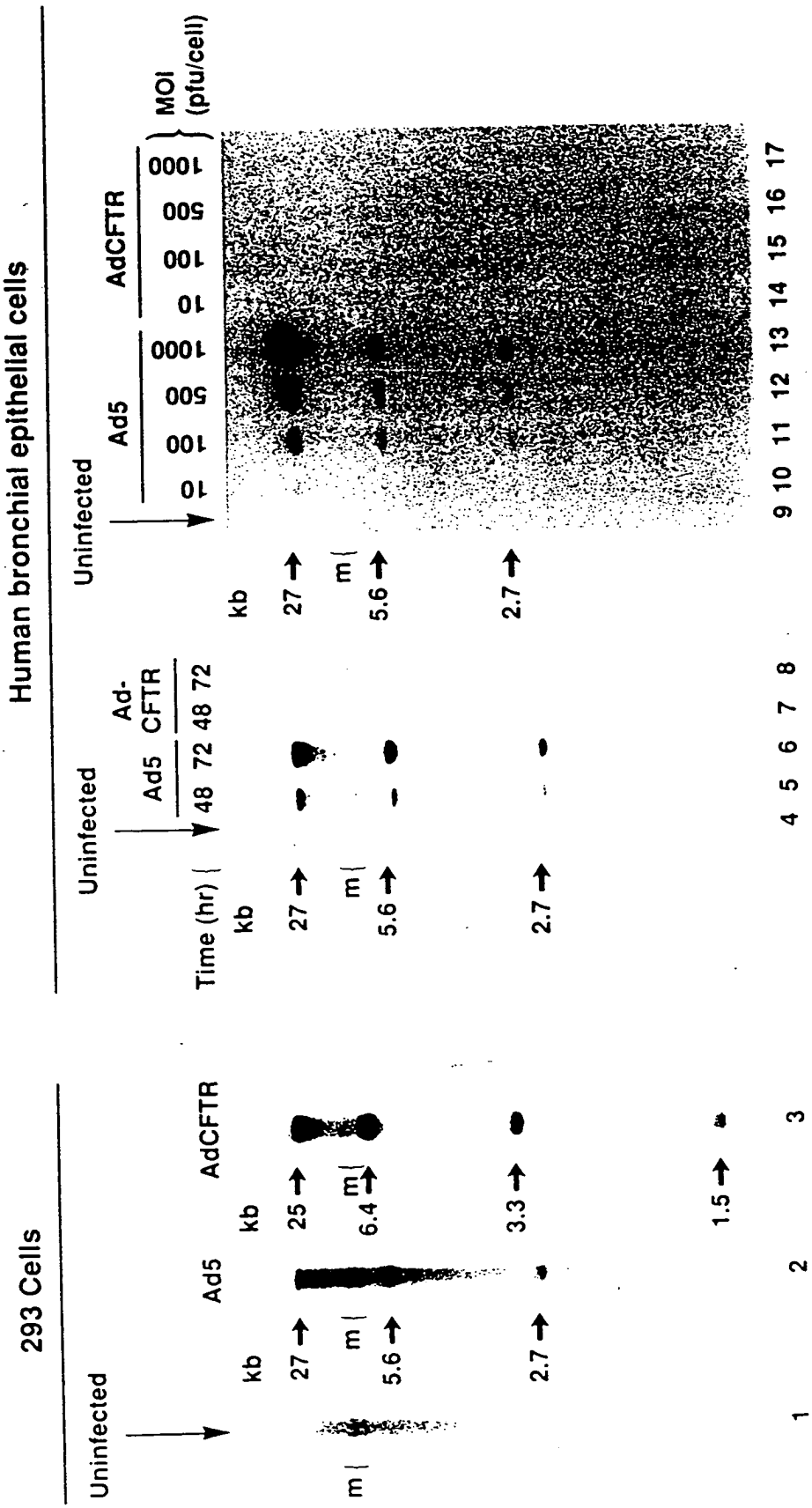

293 Cells
Uninfected
Ad5
AdCFTR
kb
27
25
m
6.4
5.6
3.3
2.7
1.5
1 2 3
Human bronchial epithelial cells
Uninfected
Ad5
Ad-CFTR
Time (hr)
48 72 48 72
4 5 6 7 8
Uninfected
Ad5
AdCFTR
MOI (pfu/cell)
10 100 500 1000 10 100 500 1000
9 10 11 12 13 14 15 16 17

Figure 4.2-A. Evaluation of human epithelial (HeLa) cells for the expression of adenoviral mRNA transcripts following infection with Av1CF1. The expression of adenovirus genes after infection of HeLa cells by the recombinant adenovirus Av1CF1 was examined for the expression of mRNA coding for the adenoviral hexon gene using northern analysis. HeLa cells were grown to 80% confluency in Dulbecco's modified Eagle medium, 10% fetal calf serum, 1% glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and infected at 100 pfu per cell. After 4 days total RNA was extracted (Chirgwin et al., 1979) and evaluated by northern hybridization analysis using as a probe a 400 bp region of the hexon gene. Ad hexon mRNA was not detected in uninfected 293 cells (lane 1) or in uninfected HeLa cells after short exposure (0.5 hr, lane 4) or long exposure (25 hr, lane 7). In contrast, Ad hexon mRNA was observed in 293 cells infected either by Ad-dl327 (lane 2) or Av1CF1 (lane 3). HeLa cells infected by Ad-dl327 showed easily detectable levels of Ad hexon mRNA on short (lane 5) and long (lane 8) exposure. In contrast, HeLa cells infected by Av1CF1 showed no Ad hexon mRNA on short exposure (lane 6) but, on long exposure (lane 9) small amounts were present.

Figure 4.2-A

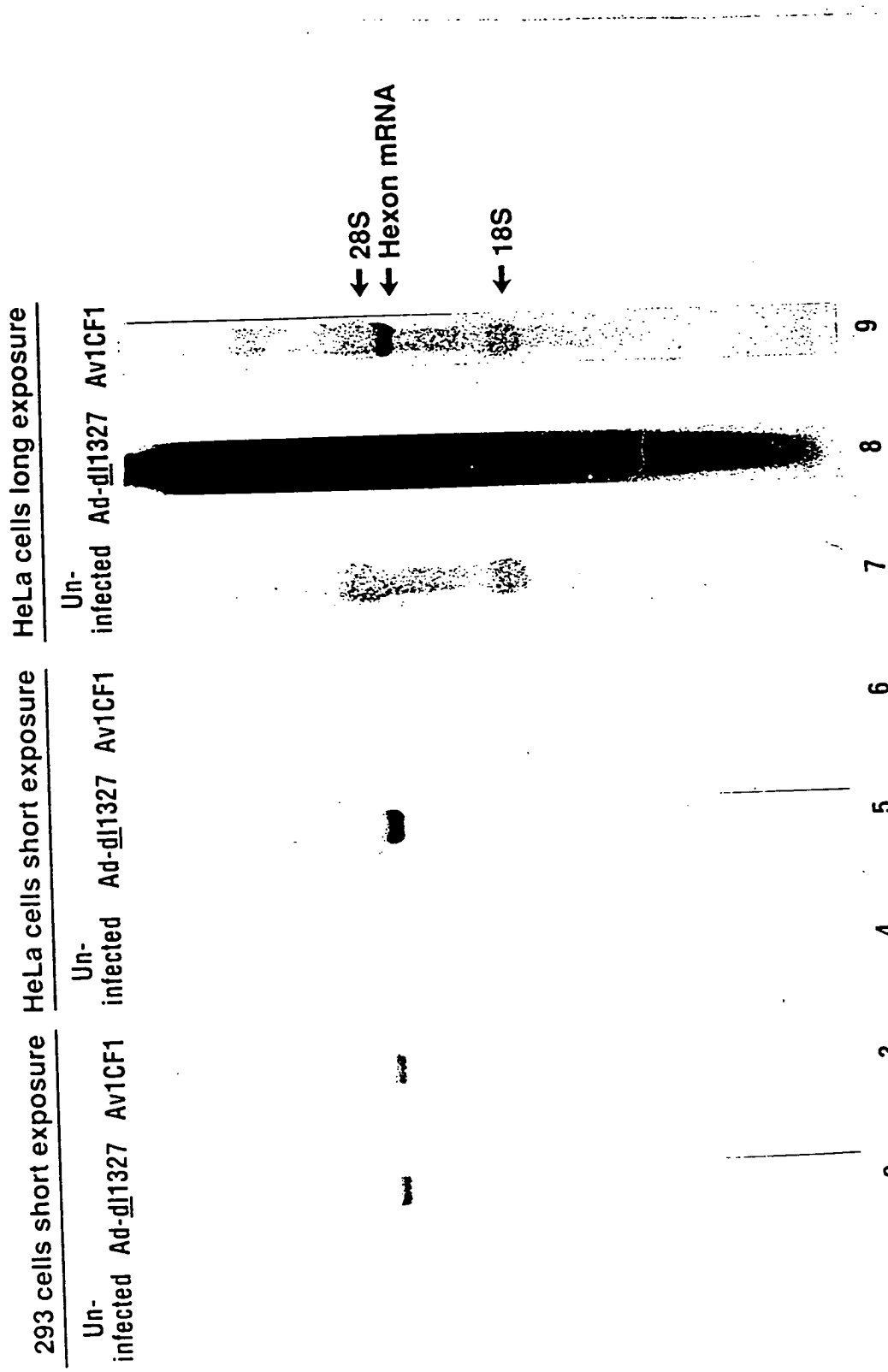
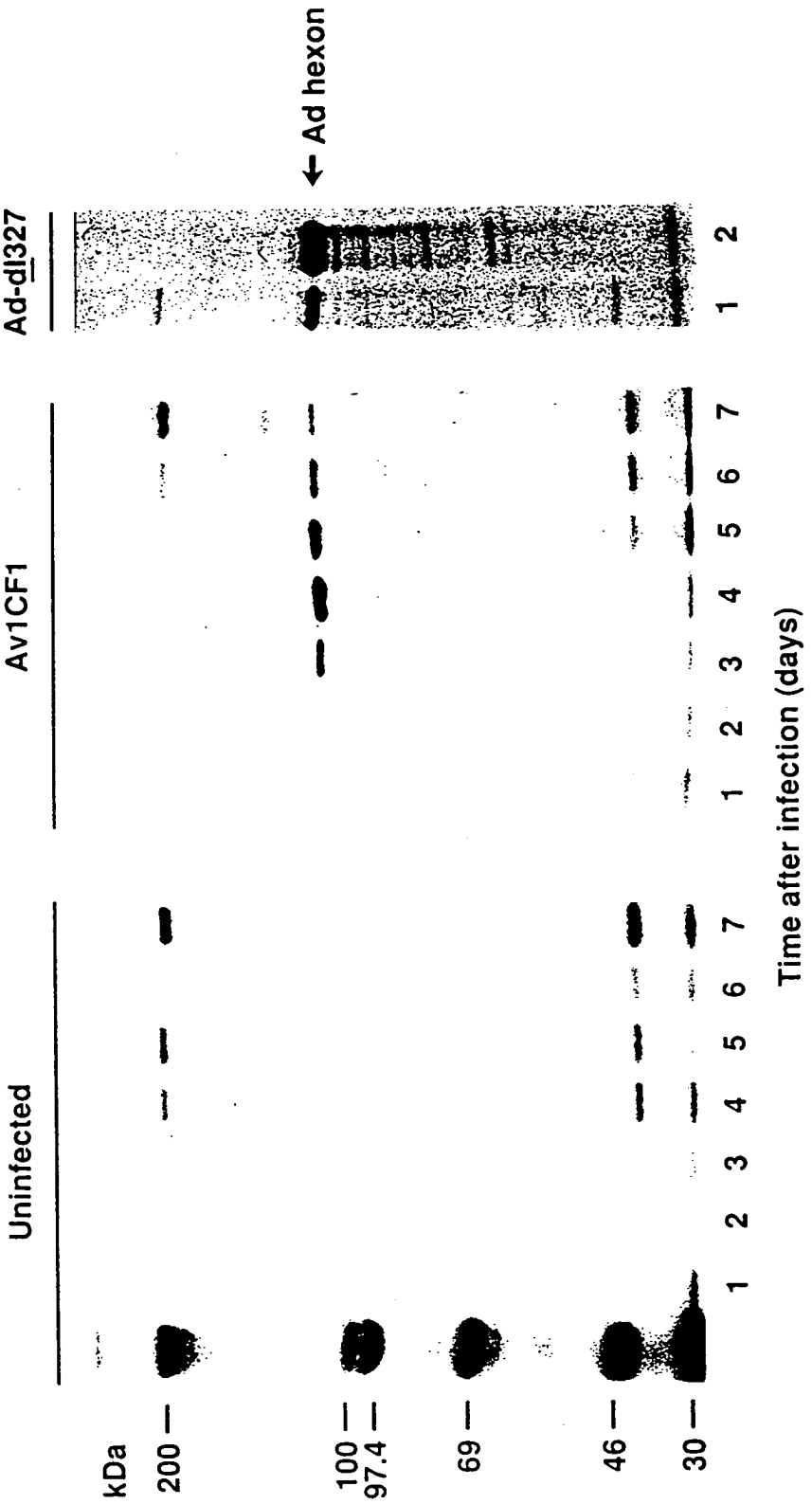

293 cells short exposure
Un-infected
Ad-dl1327
Av1CF1
HeLa cells short exposure
Un-infected
Ad-dl1327
Av1CF1
HeLa cells long exposure
Un-infected
Ad-dl1327
Av1CF1
28S
Hexon mRNA
18S
1
2
3
4
5
6
7
8
9

Figure 4.2-B. Evaluation of human epithelial (HeLa) cells for the expression of adenovirus protein following infection with AvlCFl. The expression of adenovirus genes after infection of HeLa cells by the recombinant adenovirus AvlCFl was examined at the level of protein expression by evaluating the presence of adenovirus hexon protein with and without immunoprecipitation. HeLa cells were grown to 80% confluency in Dulbecco's modified Eagle medium, 10% fetal calf serum, 1% glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and infected with AvlCFl at a multiplicity of infection (MOI) of 100 plaque forming units (pfu) per cell as previously described (Appendix 1). The cells were washed twice with cold phosphate buffered saline (PBS, pH 7.4) and labeled with [^{35}S]methionine (1000 μCi/mmole, 100 μCi/ml) for 24 hr. Following the labeling period, cells were washed in PBS, and lysed in buffer containing antiproteases (see Appendix 1 for details). The cell lysate was cleared of debris by brief (10 min) centrifugation in a microfuge. The cleared cell lysate was evaluated by immunoprecipitation using an anti-Ad5 hexon antibody (gift of Bartels, Inc.). Following immunoprecipitation of equivalent amounts of trichloroacetic acid precipitable radioactivity from each sample, labeled proteins were evaluated by sodium dodecyl sulfate acrylamide gels and autoradiography. As a control, no Ad hexon protein could be precipitated from uninfected HeLa cells collected from 1 to 7 days following infection. In contrast, after infection of HeLa cells with Ad-dl327, Ad hexon protein was readily detected early (day 1) and expression increased significantly (day 2). Further evaluations could not be made with Ad-dl327 due to lytic infection of the cells by this virus. After infection of HeLa cells with AvlCFl, no Ad hexon protein could be seen early (day 1-2, a time when abundant expression could be seen with Ad-dl327). Small amounts of expression of Ad hexon was subsequently detectable (day 3), peaked (day 4) and declined thereafter (days 5-7).

Figure 4.2-B


Ad hexon
Ad-dl327
1 2
Av1CF1
1 2 3 4 5 6 7
Uninfected
1 2 3 4 5 6 7
Time after infection (days)
kDa
200
100
97.4
69
46
30

sis (immunoprecipitation is not necessary because the hexon band is distinct on the SDS gels). In contrast, parallel studies of HeLa infected with AdCFTR showed detectable, but far less hexon biosynthesis (not shown).

Interestingly, whereas infection of freshly isolated normal human bronchial epithelial cells showed de novo hexon biosynthesis following infection with wild type (Ad5) virus, hexon biosynthesis was not detectable in parallel cultures of these cells infected with AvlCFl, i.e., if hexon gene expression does occur in human bronchial epithelium following AvlCFl infection, it is at a very low level (Figure 4.2-C). These differences between HeLa and human respiratory epithelium are consistent with the observations of AvlCFl DNA replication (section 4.1). Similar results were observed without immunoprecipitation i.e., ^{35}S-methionine labeled freshly isolated normal human bronchial epithelial cells infected with the Ad5 for 36 hr previously (labeling in the last 12 hr) showed a large amount of de novo hexon biosynthesis (immunoprecipitation is not necessary because the hexon band is distinct on the SDS gels). In contrast, parallel studies of human bronchial epithelial cells infected with AdCFTR showed no detectable hexon biosynthesis (not shown).

The conclusion from these studies is that at least one viral gene (e.g., hexon) other than the CFTR cDNA can be expressed (e.g., in HeLa cells), but at low levels, and that in the true target cells for the protocol (respiratory epithelial cells), if hexon is expressed, expression must be at a very low level.

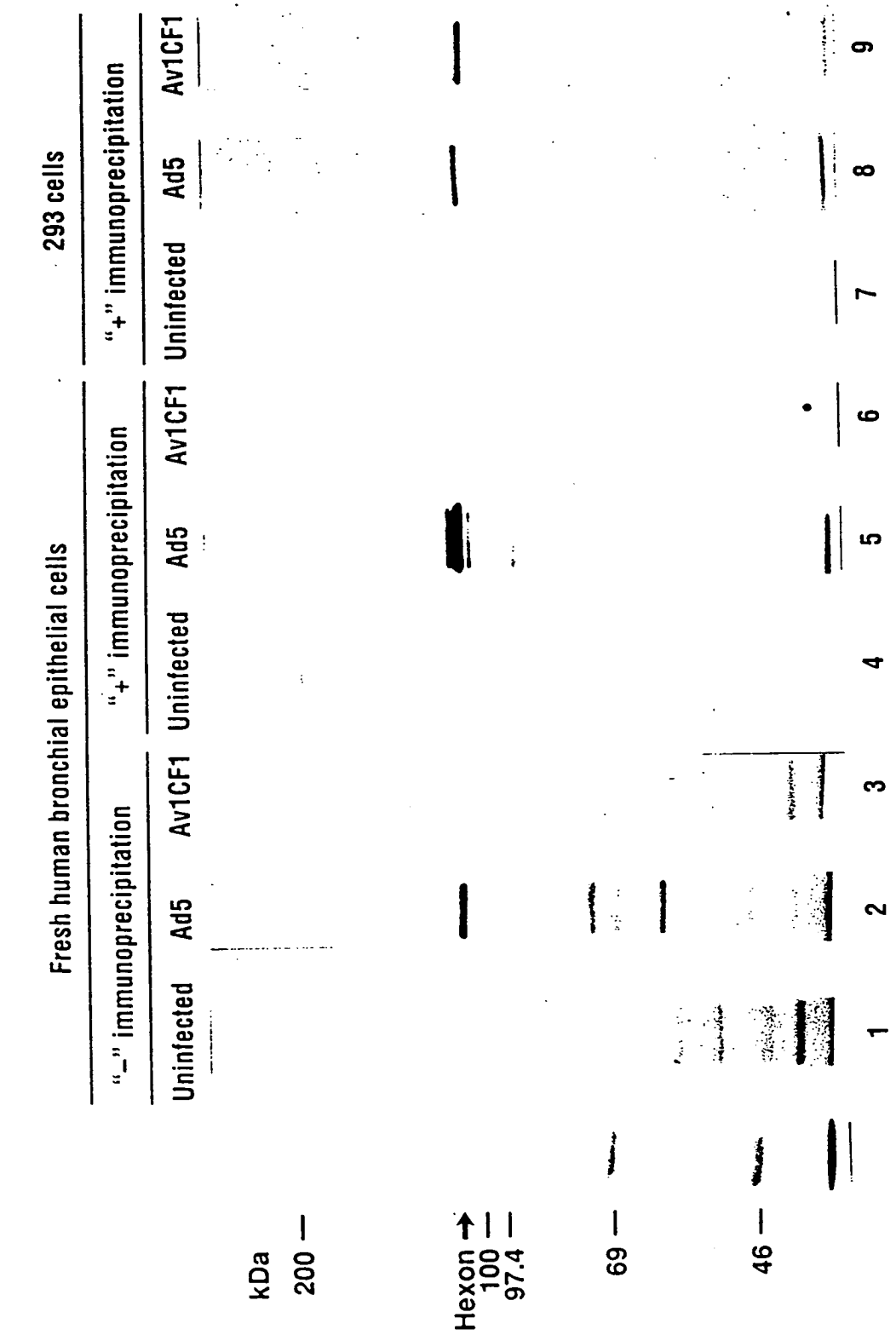
4.3 Is Respiratory Infection With the Vector Associated With Transfer of the Exogenous Gene to the Germ Line?

The purpose of this protocol is to assess somatic gene transfer to respiratory epithelial cells to treat the respiratory manifestations of CF. The endogenous CFTR gene is widely expressed, with some CFTR mRNA in almost all organs and cells evaluated, albeit at very low levels in some (Trapnell et al., 1991a; Yoshimura et al., 1991a). Expression in the gonads has been observed (Trezise et al., 1991; J. Whitsett, personal communication). From informal studies with $E1^{-}E3^{-}$ recombinant Ad-based vectors carried out to date, transfer of the vector directly to an organ (e.g., liver) has not been associated with transfer of the exogenous gene to other organs, including the gonads (Jaffe et al., 1992). Formal studies are ongoing to evaluate this with respiratory tract administration of AdCFTR.

In males with CF the question of the potential for germ line transfer is moot because almost all are sterile (Boat et al., 1989; Denning et al., 1968; Kaplan et al., 1968). To insure that germ line transmission is not possible in the males in the protocol, males will have to have documented sterility to be included in the protocol. Infertility does occur in females with CF, but it is difficult to document clinically. To insure that germ line transmission is not possible in the females in the protocol, the females to be included in the protocol will have to have definite documentation of the absence of the ovaries and/or uterus (see Section 5.4).

Figure 4.2-C. Evaluation of freshly isolated normal human bronchial epithelial cells for the expression of adenovirus protein following infection with Av1CF1. The expression of adenovirus genes after infection of human bronchial epithelial cells by the recombinant adenovirus Av1CF1 was examined at the level of protein expression by evaluating for the presence of adenovirus Ad hexon protein with and without immunoprecipitation. Human bronchial epithelial cells were obtained at bronchoscopy using a standard cytology brush as previously described (Trapnell et al., 1991a) and were cultured in suspension in Dulbecco's modified Eagle medium, 10% fetal calf serum (FBS), 1% glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and infected with Av1CF1 at a multiplicity of infection (MOI) of 100 plaque forming units (pfu) per cell in medium as above except the FBS was 2%. After 90 minutes, FBS was brought to 10% and the cells were cultured for 3 days. The cells were washed twice with cold PBS and labeled with [^{35}S] methionine (1000 Ci/mmole, 500 μCi/ml) for 24 hr. Following the labeling period, cells were washed in PBS, and lysed in buffer containing antiproteases (see Appendix 1 for details). The cell lysate was cleared of debris by brief (10 min) centrifugation in a microfuge. The cleared cell lysate was subjected to sodium dodecyl sulfate acrylamide gel electrophoresis and autoradiography. Cleared lysate was also subjected to immunoprecipitation using an anti-Ad5 hexon antibody and protein A. Ad hexon protein was not seen in uninfected cells in the absence (lane 1) or presence of immunoprecipitation (lane 4), but was abundantly expressed in cells infected by Ad5 with (lane 2) and without (lane 5) immunoprecipitation. Ad hexon protein could not be detected after Av1CF1 infection of cells either without (lane 3) or with immunoprecipitation (lane 6). As further controls, while Ad hexon could not be detected in uninfected 293 cells (lane 7), hexon was easily detected after only 1 day after infection by either Ad5 (lane 8) or Av1CF1 (lane 9).

Figure 4.2-C


Fresh human bronchial epithelial cells
293 cells
"–" immunoprecipitation
"+" immunoprecipitation
"–" immunoprecipitation
"+" immunoprecipitation
Uninfected
Ad5
Av1CF1
Uninfected
Ad5
Av1CF1
Uninfected
Ad5
Av1CF1
Uninfected
Ad5
Av1CF1
kDa
200
Hexon
100
97.4
69
46
1
2
3
4
5
6
7
8
9

4.4 Is Respiratory Administration of the Vector Associated With Damage to the Lung?

From what is known about adenoviruses in general, it is possible that the vector will damage the respiratory epithelium (or other lung components). This might take place through: (1) direct injury mediated by structural components of the virus [e.g., by penton "injury" to the cells as occurs with high MOI in culture (Everett, 1958; Pereira, 1958; Petterson, 1984)]; (2) injury mediated by replication of the virus, expression of viral genes, or expression of the CFTR cDNA; and (3) injury mediated via the inflammatory/immune system.

To evaluate the possibility of damage by an $E1^-E3^-$ recombinant adenovirus vector (through whatever mechanism), a series of studies have been carried out in cotton rats and nonhuman primates.

One time intratracheal administration of Av1CF1 (10^9 pfu/kg) to cotton rats (experiment CRC.002) resulted in no deaths over a 10 day period of observation (Av1CF1 n=17, vehicle n=15, naive n=11). Animals were sacrificed at day 3 (Av1CF1 n=6, vehicle n=5, naive n=5), or at day 10 (Av1CF1 n=3, vehicle n=2, naive n=5). Morphologic assessment at day 3 showed the Av1CF1 group had a patchy lymphocyte-dominated inflammatory reaction in the bronchoalveolar area. There was increased intensity at day 10, with inflammation localized around the vasculature, and, to a lesser extent, the airways. There were no morphologic abnormalities observed in the cells of the airway or alveolar epithelium. The vehicle group showed a very mild inflammatory reaction at day 3 which disappeared by day 10. The naive animals showed no changes.

A similar study with one time intra-nasal administration of Av1CF1 (10^{10} pfu/kg) (experiment CRCW.003) demonstrated a similar pattern over a 7 day period of observation (Av1CF1 n=26, vehicle n=22, naive n=12). There was one death in the Av1CF1 group (day 4) and one death in the vehicle group (day 5). The animals were sacrificed at day 3 (Av1CF1 n=3, vehicle n=3, naive n=3) and day 7 (Av1CF1 n=3, vehicle n=2, naive n=3). Morphologic assessment was similar to the first group of animals (experiment CRC.002) with the exception that some of the Av1CF1 infected animals also showed a very mild increase in the size of type II alveolar epithelial cells.

In another study (CRC.008), single nasal administration of Av1CF1 to cotton rats (4.3×10^{10} pfu/kg) is being compared to Ad5 (1.6×10^{10} pfu/kg). Animals will be evaluated at 1 hour and 3, 7, 14, and 28 days after infection and the two infected groups compared to naive animals in the same cages ("canary" group) as well as to animals receiving vehicle alone. After 4 weeks, there were no deaths in the Av1CF1 group (0/4 evaluated at 3 days, 0/4 evaluated at 7 days, and 0/4 evaluated at 14 days, 0/4 evaluated at 28 days). In contrast, there were 5/16 deaths in the Ad5 group. There were no deaths in the "canary" group (0/3 of the 3 day group, 0/3 of the 7 day group, 0/3 of the 14 day group, 0/3 of the 28 day group) or the vehicle group (0/2 of the 3 day group, 0/2 of the 7 day group, 0/2 of the 14 day group, 0/2 of the 28 day group). By 7 to 14 days, the animals receiving Av1CF1 showed a mild to moderate inflammatory reaction in the airways and

alveoli. However, by 4 weeks, this had resolved, with only few areas of very mild inflammation in the alveoli. In contrast, animals receiving Ad5 had inflammation, edema and epithelial damage, with mild to moderate inflammation persisting at 4 weeks. Studies are ongoing to evaluate serum antibodies and shedding (pharyngeal, rectal).

To determine if these responses to the replication deficient recombinant adenovirus were transient or permanent, in a limited, anecdotal evaluation (CR.007) of remaining animals from a prior study in which cotton rats received intratracheal Ad-CFTR 10^{11} pfu/kg, the lungs of cotton rats administered Ad-CFTR 16 weeks previously were evaluated by morphology. Consistent with the concept that the inflammatory response in cotton rats associated with respiratory administrations of Ad-CFTR (n=3) does not persist, morphologic assessment demonstrated they were no different than the lungs of animals receiving intratracheal vehicle (n=2) or a naive animal from the same group (n=1). Further evaluation of the parenchyma of these animals demonstrated no difference among the 3 groups, with no edema and/or changes in epithelial cells in either the airways or the alveoli.

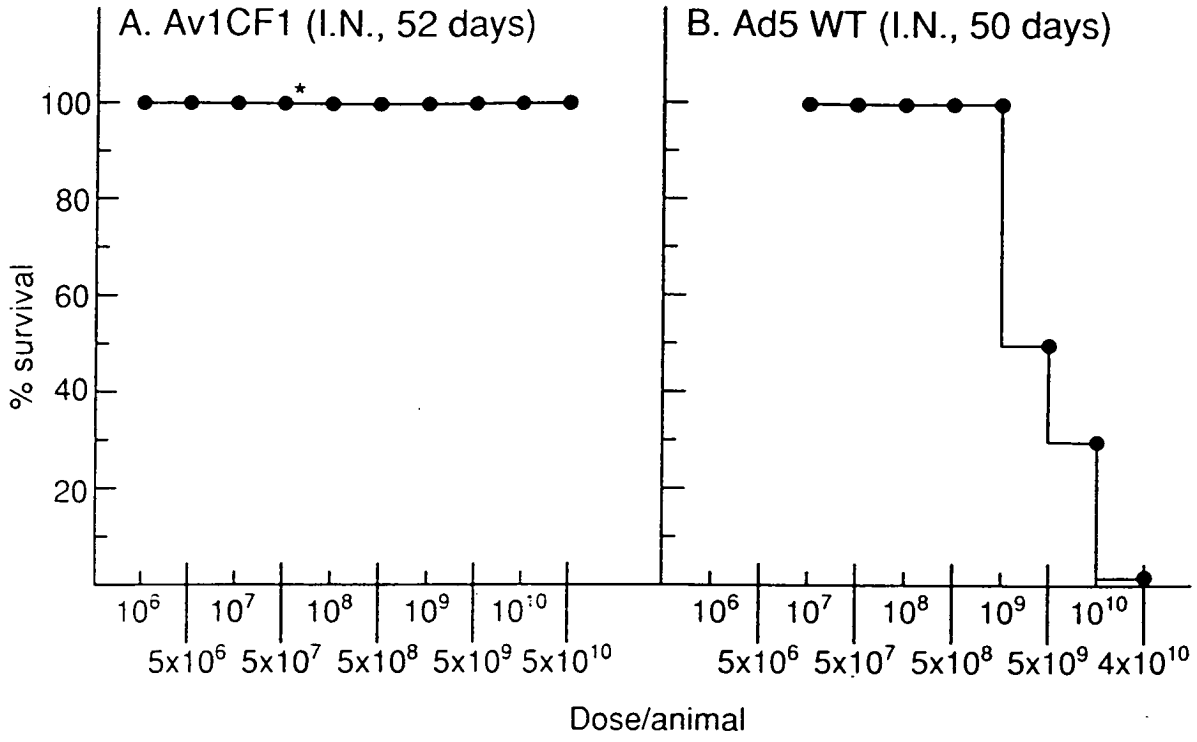
With the same batch of AdCFTR, 10^{11} pfu/kg was administered intratracheally to 4 cotton rats and in parallel, compared to vehicle (n=4) and naive animals (n=2). (CR.007A). As with intratracheal administration with AvlCFl (CRC.002), over 10 days, there were no deaths. The inflammatory response in the AdCFTR group was similar to that of several with AvlCFl (CRC.002).

To determine if a replication deficient recombinant adenovirus has lethal effects when administered to the respiratory tract of cotton rats, increasing amounts of AvlCFl were administered by the nasal route to cotton rats in comparison to Ad5 (CRLD50.010) (Figure 4.4-A). Evaluation over a period of 52 days demonstrated no deaths in cotton rats receiving 10^6 pfu/animal (n=4), $5X10^6$ (n=4), 10^7 (n=4), $5X10^7$ (n=4; except for 2 animals that died at 28d and 42d post administration following fights with other animals), 10^8 (n=4), $5X10^9$ (n=4), 10^{10} (n=4) and $5x10^{10}$ (n=4). In contrast, there were deaths in animals receiving Ad5 in a dose response fashion, as has been observed by others (G. Prince, NIAID, personal communication). The data for the Ad5 animals by day 50 included: 10^7 pfu/animal (n=4, 0 deaths), $5X10^7$ (n=4, 0 deaths), 10^8 (n=4, 0 deaths), $5X10^8$ (n=4, 0 deaths), 10^9 (n=4, 0 deaths), $5X10^9$ (n=4, 1 death day 6, 1 death day 11), 10^{10} (n=4, 2 deaths day 4, 1 death day 7), and $4X10^{10}$ (n=4, 2 deaths day 4, 1 death day 9, 1 death day 11).

From these studies it can be concluded that there are inflammatory/immune responses to respiratory administration of a replication deficient, recombinant adenovirus with the design used in this protocol. This response is not lethal. The data suggest that the inflammation does not persist, and that no chronic changes are observed in the respiratory epithelium in response to the vector.

To evaluate the recombinant adenovirus related inflammatory response observed in cotton rats in a context closer to humans, nonhuman primates were exposed via the large airways to AdRSV.βgal, a vector similar to Ad-CFTR except for the promoter (Rous sarcoma virus long terminal repeat) and the exogenous gene (E. coli lacZ coding for β-galactosidase) (see Appendix 2

Figure 4.4-A. Comparison of Av1CF1 and Ad5 for lethal effects on cotton rats following intranasal administration. A. Increasing doses of Av1CF1 were administered by the intranasal (I.N.) route and the animals followed for 52 days. B. Same for Ad5 followed over 50 days. Ordinate: % survival. The numbers of animals in each group are described in the text. * = in the Av1CF1 group, 2 animals receiving 5 x 10^7 pfu died after fights with other animals.



for details). Rhesus (5.1-8.3 kg) were exposed to AdRSV.βgal ($2\text{-}6\times10^9$ pfu/kg, n=6) or vehicle (as controls, n=3) (rhesus study 1C pre-challenge phase, 2C pre-challenge phase, and 3C). The animals have been followed for 21 to 135 days (day 21 - 2 treated, 1 control; day 28 - 2 treated, 1 control; day 135 - 2 treated, 1 control). No deaths have occurred. There were no observed changes in any animals or between the groups in: general behavior, physical exam, vital signs, complete blood counts, detailed serum chemistries, arterial blood gases, chest X-ray and lung compliance (all parameters evaluated before day 0 and at days 0, 3, 7, 14, 21, 28 and then at 2 weekly intervals). For the animals evaluated up to 21 and 28 days, bronchoalveolar lavage was carried out at day 0, 3, 7, 14, 21 or 28. There was no difference in the numbers or type of inflammatory cells recovered (animals compared to controls) and all parameters remained within the normal range.

A similar study is presently ongoing in rhesus receiving Av1CF1 (2×10^{10}/kg to the right mainstem bronchus; n=4 treated; n=1 control) (rhesus study 4C pre-challenge, 5C pre-challenge). After 3 months all animals are alive and there is no change in their general behavior, physical exam, vital signs, complete blood counts, detailed serum chemistries, arterial blood gases, chest X-ray, and lung compliance. Bronchoalveolar lavage was done on days 0, 3, 7, 14, 21, 28, 42, 56 and 83. There were no significant differences between control and treated animals in cell number or types of inflammatory cells recovered.

From the studies in nonhuman primates, it appears that no significant inflammatory response occurs on the respiratory epithelial surface following respiratory administration of a recombinant adenovirus over a 3 to 4 week period. Consistent with these observations, there are no changes in any clinical parameters including those related to the lung. Although it is possible that vector administration to the rhesus did have a non-epithelial (e.g., blood vessel or airway interstitial) inflammatory response not detected by the lavage, the lack of any abnormalities in clinical parameters suggests that there may be species to species variations in the responses to the vector. Further, if inflammatory responses do occur, they do not appear to have significant clinical relevance.

From the available data it is impossible to determine whether the individual with mild to moderate CF will respond to respiratory administration of the vector in a fashion closer to the cotton rat or to the rhesus. Further, if there is a transient inflammatory response, it should be put in context that the airways of individuals with CF are chronically inflamed, with a neutrophil-dominated inflammatory process that is very intense, far beyond that of any other chronic disorder. Quantitative assessment of this inflammation can be obtained by bronchial brush (to assess the epithelium) and bronchoalveolar lavage (to assess the inflammation in the epithelial lining fluid). The results demonstrate a marked neutrophil-dominated inflammation in the airway epithelium of individuals with CF (Table 4.4-A). It is unlikely that this ongoing chronic inflammation will be significantly influenced by a transient response to the vector should it occur.

4.5 Are Anti-Vector Antibodies Elicited by Respiratory Administration of

Table 4.4-A

Inflammatory Cells in the Airway Epithelium and in Epithelial Lining Fluid of Individuals With Cystic Fibrosis Compared to Normals[1]

Site	Group	Number of individuals	Total number of inflammatory cells recovered	Cell type (% of cells recovered)[2]			
				Neutrophils	Macrophages	Lymphocytes	Eosinophils
Nasal brush	Normal	25	19±5x10³	15±4%	1±1%	1±1%	<1%
	CF	12	49±8x10³	26±5	1±1	1±1	<1
Bronchial brush	Normal	70	18±1x10³	4±1	2±1	1±1	<1
	CF	22	562±78x10³	52±5	1±1	1±1	<1
Epithelial lining fluid	Normal	10	30±3x10³	1±1	93±1	4±1	<1
	CF	20	134±18x10³	73±5	26±5	<1	<1

[1]See Saltini et al., 1984; Trapnell et al., 1991a for methods; for epithelial lining fluid the data is presented as the number of cells recovered per microliter of epithelial lining fluid in bronchoalveolar lavage.
[2]Only inflammatory cells are listed; for the nasal brush and the bronchial brush, the remaining cells (in each row to make the total 100%) are epithelial cells.

the Vector?

There have been several studies to evaluate whether replication competent adenovirus administered to the human respiratory tract (usually nasal) will result in anti-adenovirus antibodies (see Table 5.2-A in section 5). However, although anti-adenovirus antibodies may develop, experimental animal studies suggest the respiratory route is not a very effective route to achieve immunization against recombinant proteins. For example, recent studies by B. Murphy (NIAID, personal communication) in rhesus showed that intratracheal administration of 10^8 $TCID_{50}$ of a recombinant replication competent adenovirus containing the exogenous respiratory syncytial virus (RSV) F protein gene in the E3 position elicited significant anti-adenovirus antibodies in blood, but not anti-RSV-F protein antibodies in lung.

Consistent with studies of adenovirus administration to humans, studies in cotton rats receiving intratracheal Ad-CFTR elicited anti-adenovirus antibodies in serum (Figure 4.5-A). By day 7, anti-Ad-CFTR antibodies were readily detectable in serum and remained detectable for at least 4 months. Similar studies have been carried out to evaluate serum anti-adenovirus antibodies associated with a single respiratory tract administration of AvlCFl compared to Ad5 (Figure 4.5-B). The pattern of anti-adenovirus antibodies elicited in serum was similar to that observed with respiratory administration of AdCFTR (Figure 4.5-A).

To determine the antibody response to an adenovirus type 5-based replication deficient adenovirus vector in non-human primates, a recombinant adenovirus containing the E. coli lacZ gene (AdRSV.βgal) was administered to the large airways of rhesus monkeys (rhesus study 1C and 2C). Two controls (receiving vehicle) and 4 adenovirus vector treated animals were evaluated. Adenovirus was delivered to the right mainstem bronchus at days 0 and then again at days 21 or 28. Animals were serially evaluated at day 3, then weekly for one month, then bi-weekly, then monthly, for up to 187 days. Serum antibody to adenovirus type 5 was assayed by serum neutralization of adenovirus type 5 using A549 cells. Control animals (receiving vehicle) did not have an increase in serum neutralization titers. Treated animals had the following responses: one of four animals had a marked rise in titer from <1:10 to 1:40 after 14 days; one animal did not have a rise in serum titers until after a second dose of AdRSV.βgal; the remaining two animals did not have any elevation of titer throughout the study period. All titers fell to 1:10 or lower 35 days after the second dose except in one animal in which the titer remained elevated at 1:80 at day 124. In group 3C, the control had no increase in neutralizing titer over baseline; in the 2 treated animals, 1 had no increase, and the other had a sustained increased titer of 1:10.

Similar to studies evaluating the antibody response to AdRSV.βgal, the serum neutralization titers were determined after AvlCFl (10^{10} pfu/kg) was administered to the large airways of rhesus monkeys (rhesus studies 4C and 5C). In rhesus study 4C, vehicle (n=1) or AvlCFl (n=2) was delivered to the right mainstem bronchus at day zero. Animals were evaluated weekly for 42 days, then biweekly, for up to 83 days for serum neutralization titers. The

Figure 4.5-A. Cotton rat serum anti-adenovirus antibody titers following respiratory tract delivery of Ad-CFTR, a replication deficient, recombinant adenovirus vector containing the human CFTR cDNA. Controls are presented as open symbols (□ untreated; Δ intratracheal administration of vehicle on day 0; o intratracheal administration of vehicle on day 0 and day 7). Animals receiving Ad-CFTR are presented as closed symbols (▲ intratracheal administration of Ad-CFTR on day 0; • intratracheal administration of Ad CFTR on day 0 and vehicle on day 7). Animals were sacrificed and serum obtained at the indicated time points. Anti-adenovirus antibodies were determined by enzyme linked immunosorbent assay (ELISA). Briefly, 96 well ELISA plates were coated overnight with Ad-CFTR at 4°; after washing [with phosphate buffered saline containing 0.05% Tween-20 (PBS-Tween)], serial, 4-fold dilutions of serum [in phosphate buffered saline containing 1% fetal bovine serum (PBS-1%FBS)] were added and the plates incubated overnight at 4°. Plates were washed with PBS-tween and incubated with a rabbit anti-cotton rat IgG serum (gift of Dr. B. Murphy, NIAID) for 5 hr at 4°. Plates were washed with PBS-tween and incubated with goat anti-rabbit alkaline phosphatase conjugate overnight at 4°. Plates were then washed with PBS-tween, developed for one hour with p-nitrophenyl phosphate, disodium-6-H_2O, and optical density was determined at 405 nm in an ELISA plate reader. Background, determined from samples assayed as above on wells left uncoated with virus, was subtracted and titers determined. Standardization was insured by the evaluation of standard positive and negative controls on each plate. Data is expressed as the inverse maximum dilution at which detectable signal over negative control was detected. All data points indicate the mean of two independent determinations of serum from a single cotton rat.

Figure 4.5-A

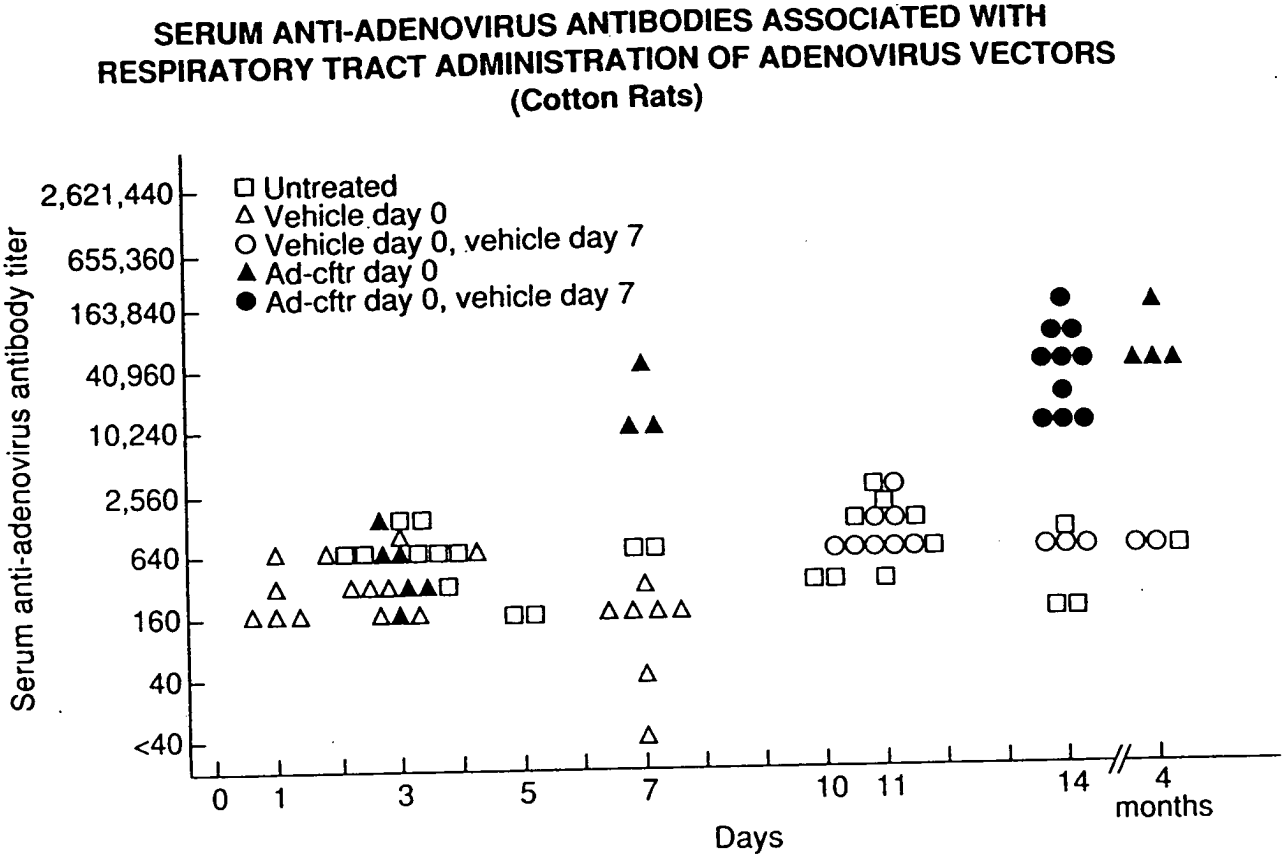
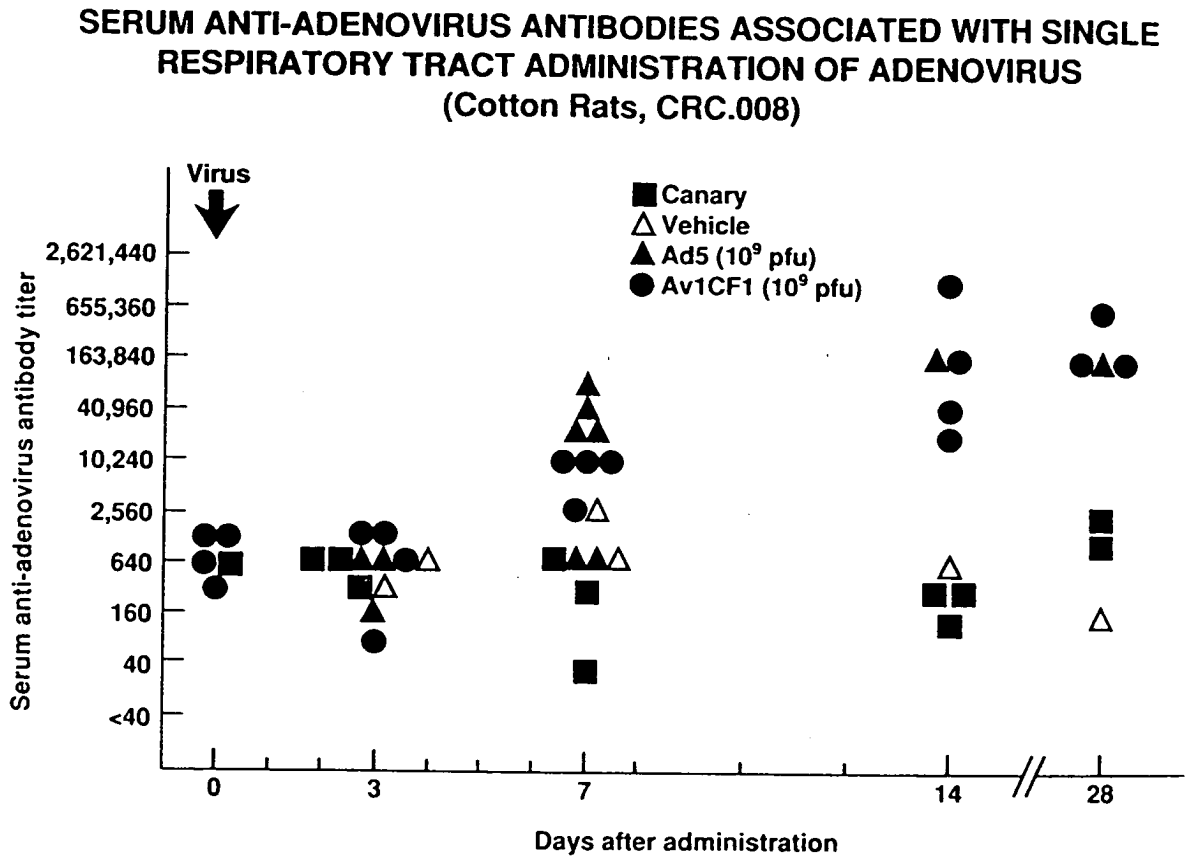

SERUM ANTI-ADENOVIRUS ANTIBODIES ASSOCIATED WITH
RESPIRATORY TRACT ADMINISTRATION OF ADENOVIRUS VECTORS
(Cotton Rats)
Untreated
Vehicle day 0
Vehicle day 0, vehicle day 7
Ad-cftr day 0
Ad-cftr day 0, vehicle day 7
Serum anti-adenovirus antibody titer
2,621,440
655,360
163,840
40,960
10,240
2,560
640
160
40
<40
0
1
3
5
7
10
11
14
4
months
Days

Figure 4.5-B. Cotton rat serum anti-adenovirus titers following nasal administration of Av1CF1 compared to Ad5. The controls included administration of vehicle and animals that are untreated but housed in proximity to the treated animals ("canary").



control animal administered glycerol containing buffer had no rise in serum neutralizing titer at any time. One of the animals receiving AvlCF1 had an increase in titer (<1:10 to 1:10) after 7 days which remained elevated (1:40) for at least 83 days. This animal had a minimally elevated titer (1:10) pretreatment which may indicate unknown prior exposure to human adenovirus, or cross-reacting virus.

In rhesus study 5C, AvlCF1 (n=2) was delivered to the right mainstem bronchus at day zero, followed by Ad 5 (10^4 pfu/kg) delivered to the mainstem bronchus at day 7. An additional 2 animals received only Ad 5 (same dose) at day 7. Serum neutralization titers did not rise in either animal administered only Ad5. However, similar to the response seen in one animal in group 2C administered a second dose of AdRSV.βgal, both animals in group 4C who received AvlCF1 followed by Ad5 did not have an increase in serum neutralization titer until after Ad5 was administered. Titers in these animals rose to 1:20 to 1:40 and remained elevated for at least 56 days, but fell to <1:10 at 83 days.

In summary, airway administration of a recombinant, replication deficient adenovirus vector results in increase in serum antibodies in cotton rats against adenovirus for at least 4 months. In rhesus, serum neutralization antibodies to Ad5 are observed after 10 days with a fall in titer after 50 days. Repeat administration of vector within one month prolongs the period of elevation of the neutralization titer. From these data, and from the accumulated data of respiratory tract administration of adenovirus in humans, it is reasonable to assume that administration of AdCFTR to the nasal and bronchial epithelium of individuals with CF will elicit antibodies in blood against adenovirus components of AdCFTR. Until the studies are carried out in individuals with CF, it is not possible to predict the local anti-AdCFTR response, if any. This is particularly true because the epithelial milieu in the CF lung is very hostile to proteins such as immunoglobulins (see Section 4.4 and references Fick et al., 1984).

If such humoral immunity is induced in the individuals receiving AdCFTR it may (or may not) have consequences for future therapy with AdCFTR. This is discussed in Section 4.6, 5.2 and 5.6.

4.6 Does Repeat Administration of the Vector Pose a Risk?

The protocol is specifically designed to evaluate a one time administration of the vector (once to the nasal epithelium, and once to the bronchial epithelium one day later), not repetitive dosing. There are several reasons for this (see Section 5.2 constraints that dictate design of the protocol). Most importantly, there is no way of estimating the dose and intervals between dosing without determining the "pharmacokinetics" of the biologic and clinical efficacy parameters following a one time administration. Further, it is conceivable that the efficacy will be long lasting and that chronic therapy will only need very infrequent intervals.

Despite this, looking toward the future, it is relevant to ask whether repeat administration of the vector poses a risk to the individual should this study demonstrate it will be required in the future.

To begin to evaluate this question, cotton rats were administered AvlCF1 (intratracheal 10^9 pfu/kg). Ten days later, the animals were challenged with the same dose of intratracheal AvlCF1, and 4 days later the animals were sacrificed (AvlCF1 followed by AvlCF1 n=8; vehicle followed by vehicle n=8) CRCC.002. No deaths were observed. The morphology of the lungs in the AvlCF1/AvlCF1 group showed a mononuclear cell inflammatory response, but somewhat milder than that of the animals evaluated at the beginning of the challenge period (i.e., 10 days after a single administration of AvlCF1, see Section 4.4). The animals receiving the vehicle had no inflammation.

These studies suggest that the intratracheal challenge with the vector would not result in an adverse reaction with severe acute clinical consequences. Studies are ongoing to determine if the observed inflammatory response will persist or it will disappear over time.

Evaluation of an ongoing challenge study of rhesus suggests that the inflammation response may disappear over time. Four rhesus (5.1-8.3 kg) were administered the AdRSV.βgal vector to the lung ($2\text{-}8\times10^9$ pfu/kg via a bronchoscope to the large airways) (rhesus study 1C and 2C). Animals were followed for 21 to 28 days and then re-challenged with the same vector, same dose, in the same location. Two control animals (vehicle followed by vehicle 21 or 28 days later) were evaluated in parallel. These 6 animals (4 with repeat treatment, 2 control) have been followed for 180 to 187 days to date. No deaths or complications have occurred. Animals were followed with all of the parameters described in section 4.4; no abnormalities were observed in any parameter including the chest X-ray, arterial blood gases and lung compliance. Bronchoalveolar lavage of the 2 control animals showed no changes in total cells recovered or cell differential. In the 4 animals treated with vector and then challenged with the vector, the challenge was followed by an increase in cell number and proportion of lymphocytes. The data to date suggest the inflammatory response is resolved or resolving with return to baseline cell number, although the proportion of lymphocytes remain elevated.

From these data, it can be concluded that re-challenge with the same recombinant vector (should it be necessary), will not be expected to have severe, acute clinical consequences. Studies are ongoing to determine the long term status of these animals.

4.7 Is the Vector Shed Following Respiratory Administration of the Vector?

A series of studies have been carried out which demonstrate that shedding occurs infrequently, and only at early times after administration of $E1^-E3^-$ recombinant adenovirus administration.

First, in cotton rats, intranasal administration of AvlCF1 (10^{10}pfu/kg) followed by wild type Ad5 (10^9/pfu/kg) one week later (CRCW.003), evaluation of pharyngeal and rectal swabs for the presence of adenovirus revealed that:

	at 7 days # positive for adenovirus		at 14 days # positive for adenovirus	
	pharynx	rectum	pharynx	rectum
vehicle at 0 time	0/3	0/3		
vehicle at 0 times, vehicle at 7 days			0/2	0/2
vehicle at 0 time Ad5 at 7 days			2/4	0/4
AvlCF1 at 0 time	0/3	0/3		
AvlCF1 at 0 time, vehicle at 7 days			0/5	0/5
AvlCF1 at 0 time, Ad5 at 7 days			0/3	0/3

Thus, while exposure to Ad5 alone resulted in Ad5 shedding 7 days later, pre-exposure to AvlCF1 did not. This is consistent with the concept that $E1^-E3^-$ adenovirus vectors will suppress wild type replication (see Section 4.9) and that it is not a danger to the environment. To evaluate shedding of recombinant replication deficient adenovirus vector (AdRSV.βgal) in rhesus, adenovirus was administered to airways of 3 groups of rhesus monkeys (rhesus study 1C, 2C and 3C), each group composed of 1 control receiving vehicle) and 2 adenovirus treated animals. Groups 1 and 2 had adenovirus ($2\text{-}8\text{x}10^9$ pfu/kg) delivered to the right mainstem bronchus on days 0 and 21 or 28. Group 3 has adenovirus delivered to the trachea (and held in place by balloon tamponade for 30 minutes) on day 0 and day 135. Animals were serially followed (weekly for one month, then bi-weekly, then monthly) for shedding for up to 187 days by obtaining whole blood, lower airway lavage with sterile saline, and swabs from posterior pharynx and rectum. To detect adenovirus, samples were cultured on 293 [which allows the growth (and hence detection) of adenovirus with or without the Ela region] and A549 cells (which allows the growth only of adenovirus containing the Ela region, i.e., replication competent adenovirus). The recombinant Ela deficient adenovirus vector (but not replication competent adenovirus) was detected in secretions of 1 animal in groups 1 and 2; on day 3 in fecal samples and lung lavage, and day 43 (15 days after receiving a second dose of the same adenovirus) in lung lavage only. In group 3, Ela deficient adenovirus was detected in the lung lavage and rectum fecal samples of 2 treated animals at day 3 after which it was not detected during a 46 day follow-up period.

Adenovirus shedding following AvlCF1 administration was also evaluated. As with AdRSV.βgal, AvlCF1 ($2\text{X}10^{10}$ was administered to the large airways of rhesus monkeys (rhesus studies 4C and 5C). In rhesus study 4C, vehicle (n=1) or AvlCF1 (n=2) was delivered to the right mainstem bronchus at day zero. Animals were evaluated weekly for 42 days, then biweekly, for up to 56 days for the presence of adenovirus in blood (days 0-7 only), respirato-

ry lavage fluid, posterior pharynx and rectal swab samples using the culture techniques described in the previous paragraph. Neither control animal administered vehicle or the animals administered AvlCFl, had adenovirus detectable in any sample (data analyzed to 42 days post-administration).

In rhesus study 5C, AvlCFl (n=2) was delivered to the right mainstem bronchus at day zero, followed by Ad 5 (10^4 pfu/kg) delivered to the mainstem bronchus at day 7 and an additional 2 animals received only Ad 5 (same dose) at day 7. Samples were obtained for evaluation as described for group 4C. Adenovirus could not be detectable in any samples from these animals (data analyzed to 35 days post-administration).

In summary, replication deficient adenovirus was infrequently detected in secretions, typically at less than one week after administration and no longer than 15 days after administration.

To evaluate the possibility that respiratory administration of a recombinant vector would gain access to the gastrointestinal tract and be subsequently shed [something observed with replication competent Ad infection in which fecal shedding is typically observed (Fox et al., 1969)], rhesus (n=3; rhesus study El) were given a replication deficient recombinant adenovirus containing the human α1-antitrypsin cDNA (see Rosenfeld et al., 1991 for details) in enteric release capsules (10^8-10^9pfu/kg, n=3 rhesus, 3 total doses each).

The capsules were administered to 3 animals housed in open caging in the presence of 2 non-treated, sentinel animals. Sentinel animals were housed next to or across from treated animals. All animals were in the same room maintained under negative airflow (relative to the hall), and a dry bedding system, changed three times weekly, was used. Recombinant adenovirus, in capsules designed for enteric release, was administered on days 1,4 and 7. Secretions (posterior pharynx and rectum fecal samples) for adenovirus detection (by culture on 293 cells) were sampled from treated and sentinel animals one day after each dose, and at days 14, 28, and 43. All treated animals had adenovirus detected by culture from secretions (oral and/or fecal) one day after at least two of the adenovirus doses. However, no sentinel animal had adenovirus detected in secretions at any time. This suggests that inadvertent transfer of a recombinant virus from a treated to an untreated individual via this route is unlikely.

4.8 Are there Risks of Having Some of E3 Deleted From the Vector?

The Ad5 E3 region includes 3456 bp (nucleotides 27576 to 31032, map units 76.6 to 86.2) and codes for at least 6 proteins. In AvlCFl (which is based on Add1327), and AdCFTR (which is based on Add1324), 66% of E3 is deleted (nucleotides 28,592 to 30,470 in Ad5; map units 78.4 to 84.7). In wild type adenovirus, the E3 mRNAs arise by alternative splicing of a common precursor with two polyadenylation sites, E3A and E3B (Cladaras and Wold, 1985; Chow et al., 1979; Wold and Gooding, 1991). The E3A products include: gp19K, 11.6K, 6.7K, and 3.6K. The E3B products include: 7.5K, 10.4K, 12.5K, 14.5K, and 14.7K. The predicted 3.6K, 7.5K and 12.5K products have not been identified.

The question regarding the safety of deleting the E3 genes is related to the function of their products relevant to subverting the inflammatory/immune system in recognizing the virus i.e., does the deletion of gp19K, 10.4K and 14.5K genes in AdCFTR and Av1CF1 have relevance from a safety view point?

The gp19K product is a transmembrane protein with two N-linked glycosylation sites. It is localized in the endoplasmic reticulum of Ad-infected cells where it forms non-covalent complexes with newly synthesized class I MHC antigens, blocking the transport of these antigens to the cell surface (Kvist et al., 1978; Wold and Gooding, 1991). This has the effect of suppressing cytotoxic T-cell responses. Studies in cotton rats have demonstrated that nasal administration of a replication competent adenovirus type 2 with a deletion of the gene coding for the gp19K protein was associated with increased lymphocyte/macrophage inflammatory response in the lung compared to infectious, replication competent Ad2 (Ginsberg et al., 1989).

The 10.4K and 14.5K products are integral cytoplasmic membrane proteins co-translated from the same mRNA (Tollefson et al., 1990a; Tollefson et al., 1990b; Wold and Gooding, 1991). Both function to down regulate the epidermal growth factor receptor (EGF-R) in human Ad-infected cells (complexes of these proteins induce endosome-mediated degradation of EGF-R) (Carlin et al., 1989; Tollefson et al., 1991). In the context that this mimics the normal function of EGF, it has been suggested that this activates the target cell, and may help the virus to replicate. Both proteins block the effects of tumor necrosis factor of mouse cell lines (Gooding et al., 1988; Gooding et al., 1991).

The advantages of deleting the middle portion of the E3 region are as follows:

(1) If recombination should occur (for example a cross over to produce a infectious, replication competent $E3^-$ adenovirus), it is desirable to have the immune/inflammatory system recognize the infected cells and suppress the infection. Since E3 products subvert the ability of the immune/inflammatory system to recognize adenovirus, the inclusion of E3 may subvert the ability of the immune/inflammatory system to clear a replication competent recombinant adenovirus. In this context, if a replication competent recombinant adenovirus should emerge, it is an advantage to have the immune/inflammatory system recognize this and respond to it appropriately to clear the infection.

(2) If complementation should occur, the result would be more AdCFTR, a virus identical to the vector used for therapy.

(3) The CF airways are constantly exposed to an intense inflammatory process, likely outweighing any process induced by emergence of an $E3^-$ replication competent adenovirus (see Section 4.4 and Table 4.4-A).

(4) Human studies with enteric administration of a wild type $E3^-$ type

7 based Ad in which the hepatitis surface B antigen was inserted into the E3 region demonstrated that the $E3^-$ recipients shed less virus than the wild type virus (Tacket et al., 1992).

(5) Studies of co-infection of cells in vitro with an $E3^+$ and $E3^-$ adenovirus demonstrate that the $E3^-$ mutant suppresses the growth of the wild type $E3^+$ Ad (see Berkner and Sharp, 1983 and section 4.9).

In conclusion, it is preferable not to allow the virus to subvert these inflammatory/immune related processes associated with clearing replication competent adenovirus. Further, the limited human data available suggests the $E3^-$ deletion in a replication competent adenovirus is associated with less shedding, i.e., an $E3^-$ vector is likely safer for the individual and the environment than is an $E3^+$ vector.

4.9 Can Recombination or Complementation of the Vector Occur In Vivo Following Respiratory Administration of the Vector, and If So, Does This Pose a Risk to the Patient and/or Environment?

Although AvlCFl and AdCFTR will not replicate in freshly isolated human airway epithelial cells (see Sections 2.3, 4.1), the fact that the vectors are fabricated in a fashion that uses complementation to produce the final replication deficient recombinant vector implies that the same could occur in vivo in respiratory epithelial cells following administration of the vector. To demonstrate that complementation of a vector of this design can occur in human epithelial cells, HeLa cells were co-infected in vitro with AvlCFl and replication competent Ad5. Whereas AvlCFl DNA did not replicate at an MOI of 30 pfu/cell, addition of Ad5 resulted in de novo synthesis of AvlCFl DNA (Figure 4.9-A). The same phenomenon of complementation has been observed in freshly isolated normal human airway epithelial cells as infected with AvlCFl followed by Ad5 (not shown). Thus, under the right circumstances, complementation can occur.

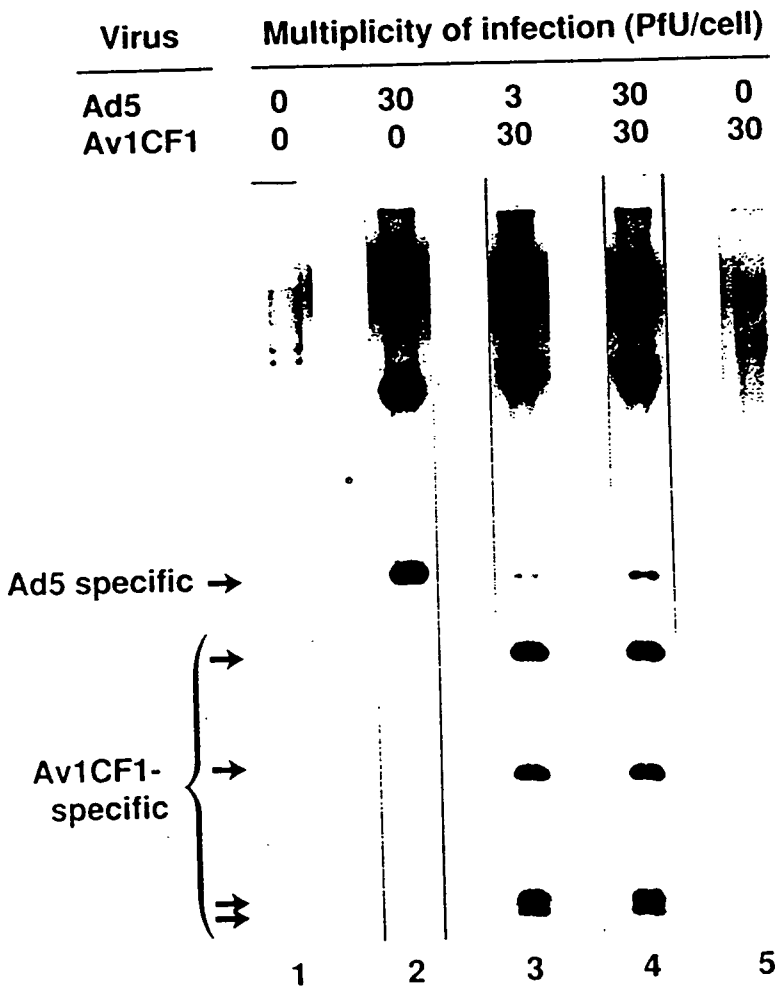
In regard to recombination, the vector design is such that a recombinational event including the intact CFTR cDNA in a replication competent virus is highly unlikely because of the difficulty in packaging such a viral genome (Ghosh-Choudhury et al., 1987). It is theoretically possible that the 5′ end of the large CFTR cDNA could be packaged in such a fashion, but this would likely be akin to a stop codon or splice junction mutation in CFTR, a circumstance that produces no protein [and usually milder respiratory disease than the common CFTR mutations (Cutting et al., 1990; Hamosh et al., 1991)].

If complementation were to occur, the result would be more AdCFTR. This should not be a problem in terms of over-expression (see Section 4.12), although the consequences of an increased dose of vector per se is not known (see Section 4.4). Alternatively this could be viewed as positive from the therapeutic point of view, in that chronicity of expression, and hence chronicity of efficacy, might be extended.

If recombination were to occur, the overwhelming likely result is a repli-

Figure 4.9-A. Evaluation of co-infection of AvlCF1 and wild-type adenovirus in HeLa cells. To evaluate the possible complementation of AvlCF1 with potentially co-infecting wild-type adenovirus, HeLa cells were simultaneously infected by two viruses. HeLa cells were grown to 80% confluency in Dulbecco's modified Eagle medium, 10% fetal bovine serum, 1% glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and infected with AvlCF1 at various multiplicities of infection (MOI) from 0 to 30 plaque forming units (pfu) per cell (Berkner and Sharp, 1983). After incubation for 20 hr (37°, 5% CO_2, humidified atmosphere), the media was aspirated and replaced with similar media except without phosphate and containing ^{32}P-$PO_4^{=}$ and the incubation was continued for another 20 hr. Cells were then lysed and incubated (37°, with shaking) in 0.6% sodium dodecyl sulfate, 10 mM Tris-HCL, pH 7.4) 10 mM EDTA, 5 mg/ml proteinase K and viral DNA was extracted by the Hirt procedure (Hirt, 1967). Equal volumes of Hirt extracted DNA were then subjected to EcoRI enzyme cleavage and the products were fractionated on a 1% agarose gel which was dried and subjected to autoradiography. Uninfected HeLa cells showed no labeled viral DNA fragments, but did show labeling of mitochondrial DNA (lane 1). HeLa cells infected with Ad5 alone demonstrated the replication of Ad5 DNA as evidenced by the presence of characteristic restriction fragments of ^{32}P-labeled Ad5 DNA (lane 2). Co-infection of Ad5 (MOI = 3) and AvlCF1 (MOI = 30) (lane 3) and Ad5 (MOI = 30) and AvlCF1 (MOI = 30) (lane 4) demonstrated replication of both viral genomes as evidence by the presence of characteristic ^{32}P-labeled restriction fragments for each virus. No replication of AvlCF1 was observed after infection of HeLa cells alone (lane 5). Interestingly, the replication of Ad5 in the presence of co-infecting AvlCF1 was diminished compared to infection by Ad5 alone (compare lanes 3,4 to lane 2).

Figure 4.9-A


Virus
Multiplicity of infection (PfU/cell)
Ad5
Av1CF1
0
0
30
0
3
30
30
30
0
30
Ad5 specific
Av1CF1-
specific
1
2
3
4
5

cation competent adenovirus that is $E3^-$ (Figure 4.9-B). The chances of this occurring in the respiratory tract following AdCFTR administration in humans with CF is not possible to estimate. However, there are ways to minimize this from occurring. Further, if this should occur, the outcome will likely be no worse and possibly less serious, than that of an infection with a naturally occurring adenovirus.

First, the Ela sequences would have to be exogenous, as the individuals with CF will be screened to insure that they are Ela^- in their respiratory tract. To form the basis of this concept, individuals with CF and controls were evaluated for the presence of Ela (Table 4.9-B). Importantly, only 10% were positive; of these individuals, there was an average of 87 ± 26 copies of Ela/10^3 airway epithelial cells (Table 4.9-B). The concept of using Ela^- epithelia as a criteria for entry into the protocol is discussed in further detail in Section 5.3.4.

Second, to further insure that the Ela sequences would have to be exogenous, the individuals with CF will be screened to insure that they are not harboring active respiratory viruses in the respiratory epithelium. Screening of such patients demonstrates that harboring such viruses is rare (only herpes simplex was found among several virus types, and herpes simplex was only found in 2 of 29 individuals, Table 4.9-C). Thus, negative cultures for active virus in the respiratory tract will be used as an inclusion criteria for the protocol (see Section 5.3.2).

Third, to minimize Ela sequences from exgenous sources (e.g., adenovirus infection), the individual will be kept under gown and glove respiratory precautions (see Section 5.6) for 10 days before and after therapy, and insuring no contact with children under the age of 15 (the most likely ages for transmission of respiratory viruses). Further, no individual will be treated that has signs or symptoms of a respiratory tract infection.

Fourth, replication competent wild type $E3^-$ adenovirus has been used in humans in vaccine trials (see Section 4.8), with actually less shedding than with the wild type $E3^+$ adenovirus.

Finally, an $E1^-E3^-$ Ad5-based vector containing the human CFTR cDNA suppresses the replication of replication competent Ad5 (Figure 4.9-A), similar to that observed with an $E3^-$ replication competent adenovirus co-infected with an $E3^+$ replication competent adenovirus.

Ongoing studies with cotton rats and rhesus are being carried out to determine the consequences of exogenous replication competent adenovirus being made available to the respiratory epithelium of experimental animals previously treated with an $E1^-E3^-$ adenovirus vector, i.e., are there adverse clinical consequences of exposure to replication competent adenovirus in the circumstance where there has been prior administration of a vector of this design?

Cotton rats were treated with AvlCF1 (intranasal 10^{10} pfu/kg). After 1 week, they were then exposed to Ad5 (10^9 pfu/kg) (study CRCW.003). In the group treated with AvlCF1 followed by Ad5, 8/9 animals survived over the 3

Figure 4.9-B. Theoretical recombination of AdCFTR with Ad5. At the top is a schematic of AdCFTR (see section 2, figures 2.3-A, 2.3-B and appendix 1 for details). Below that is a schematic of Ad5. If the genomes of AdCFTR and Ad5 were in the same cell and a homologous recombination event took place, the most likely product would be a replication competent Ad5-based virus with an E3 deletion. This is the same as the Add1327 E3⁻ Ad5 virus (see section 2 for details).

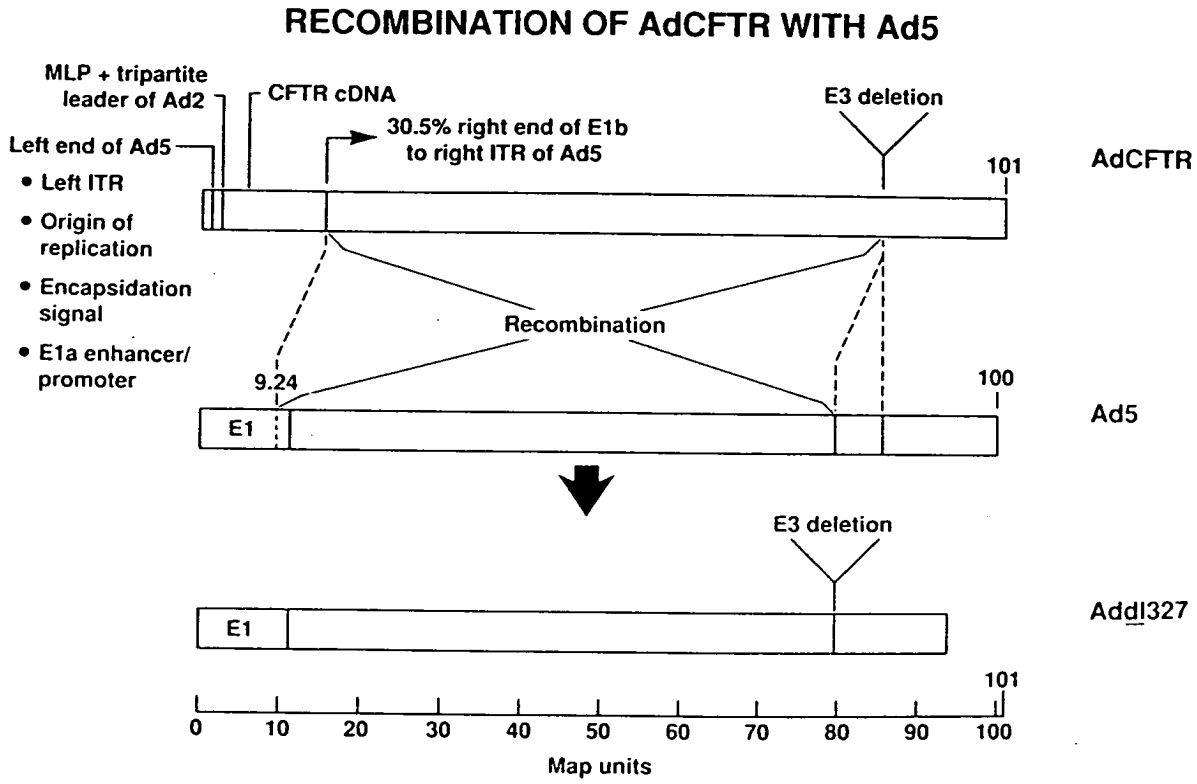


Table 4.9-B. Detection of Ela sequences in the respiratory epithelium of individuals with cystic fibrosis. Respiratory epithelial cells were obtained from normals and individuals with cystic fibrosis (CF) from the inferior turbinates of the nose (by direct visualization) and bronchial epithelial cells from trachea and/or main bronchi by fiberoptic bronchoscopy, using a standard cytology brush. Neutrophils, lymphocytes and monocytes were isolated from the blood. Cells were immediately suspended in RPMI 1640, collected by centrifugation and lysed in 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M β-mercaptoethanol. Cell number was determined in the cell lysate by quantifying the number of Alu sequences present in the DNA in an aliquot of the cell lysate compared with a standard curve of highly purified human genomic DNA (assuming 7.2 pg of DNA per cell). To detect Ela sequences, the DNA in an aliquot of cell lysate was amplified by the polymerase chain reaction (PCR) using Taq DNA polymerase and Ela specific primers (Ad17: 5'-GAGACATATTATCTGCCACGGAGG-3' and Ad18: 5'-TTGGCATAGAAACCGGACCCAAGG-3'; the sequences of both primers are 100% homologous to type 2 and type 5 adenoviruses) for 35 cycles (94°-1 min, 65°-1 min, 72°-1 min) (Gingeras et al., 1982; Van Ormondt et al., 1978). In parallel with each PCR amplification, a standard curve was constructed using increasing amounts of adenovirus type 2 DNA. An aliquot of PCR product of each sample was bound to slot-blot nylon filter and hybridized with a ^{32}P-labeled "nested" Ela probe. A total of 91 normal subjects and 43 individuals with CF were studied.

Table 4.9-B

DETECTION OF Ela SEQUENCES IN FRESHLY ISOLATED RESPIRATORY EPITHELIUM

Site	Diagnosis	Number of subjects	Ela Positive [number (%)]	Number of Ela copies per 1000 cells
Respiratory epithelium*	Normal	91	19 (21%)	55 ± 18**
	CF	43	5 (12%)	104 ± 22
Blood Cells				
Neutrophils	Normal	17	0	
	CF	5	0	
Lymphocytes	Normal	16	3 (19%)	51 ± 8
	CF	5	0	
Monocytes	Normal	16	0	
	CF	5	0	

* Nasal, pharyngeal, tracheal and bronchial epithelium.

** Mean ± SEM.

Table 4.9-C. Assessment of nasal epithelium, bronchial epithelium, and inflammatory cells on the respiratory epithelial surface of individuals with cystic fibrosis for the presence of respiratory viruses. Individuals with stable cystic fibrosis (not during an exacerbation of respiratory symptoms), normals, individuals with α1-antitrypsin deficiency, and individuals who smoke cigarettes (but are otherwise normal) underwent fiberoptic bronchoscopy with sampling of respiratory epithelium by brushing nasal mucosa, brushing large airways (bronchial epithelium), or bronchoalveolar lavage (to recover inflammatory cells on the respiratory epithelial surface) by standard techniques. Samples were evaluated for virus by culturing on tissue culture cell lines in the presence of antibiotic and antifungal suppression. Specific viruses were identified by growth on the appropriate cell lines and confirmed by specific immunofluorescent staining for virus specific capsid proteins with FITC-conjugate anti-viral capsid monoclonal antibodies. Viruses evaluations included: cytomegalovirus (CMV), varicella-zoster virus (VZV), herpes simplex viruses I and II (HSV), influenza viruses A and B (INF A,B), parainfluenza viruses 1, 2 and 3 (PARA 1,2,3), respiratory syncytial virus (RSV), and adenovirus (ADENO, including all human non-enteric types). All samples were evaluated in duplicate. Note that "INF A,B, + PARA 1,2,3" are detected by one culture system, while "RSV, ADENO + PARA 1,2,3" are detected by another culture system. Of all samples evaluated, virus was recovered from only 2 individuals with cystic fibrosis, with herpes simplex virus recovered from bronchial epithelium and lavage of one individual and lavage alone from a second individual. All other respiratory samples were culture negative for all viruses.

Table 4.9-C

Assessment of Nasal Epithelium, Bronchial Epithelium, and Inflammatory Cells on the Respiratory Epithelial Surface of Individuals with Cystic Fibrosis for the Presence of Respiratory Viruses

		(Number of + samples/number of samples evaluated)				
Study group	Site	CMV	VZV	HSV	INF A,B PARA 1,2,3	RSV, ADENO, PARA 1,2,3
Cystic fibrosis	Nasal	0/21	0/21	0/21	0/20	0/20
	Bronchial	0/31	0/30	1/30	0/29	0/29
	Lavage	0/29	0/26	2/29	0/26	0/26
Normal	Nasal	0/22	0/22	0/22	0/21	0/21
	Bronchial	0/22	0/22	0/22	0/20	0/20
	Lavage	0/22	0/22	0/22	0/20	0/20
α1-antitrypsin	Nasal	0/6	0/6	0/6	0/6	0/6
	Bronchial	0/7	0/7	0/7	0/7	0/7
	Lavage	0/10	0/10	0/10	0/10	0/10
Cigarette smokers	Nasal	0/3	0/3	0/3	0/3	0/3
	Bronchial	0/3	0/3	0/3	0/3	0/3
	Lavage	0/3	0/3	0/3	0/3	0/3

to 7 day observation period. Of animals receiving vehicle followed by vehicle, (6/6) survived over the 3 to 7 day period, as did naive animals (6/6), animals receiving vehicle alone (10/10), and animals receiving vehicle followed by Ad5 (10/10). Studies with a larger group of animals (study CRCW.009) are ongoing to evaluate dosing and timing.

Studies are also ongoing in rhesus with n=2 receiving AvlCFl (intrabronchial $2x10^{10}$ pfu/kg) followed 1 week later with Ad5 (intrabronchial 10^{4} pfu/kg) (rhesus study 4C and 5C). Controls include Ad5 alone (n=2), AvlCFl alone (n=2) and a naive animals (n=1). All are alive and well 83 days after administration of the wild type Ad5 virus. All cultures for adenovirus in A549 and 293 cells are negative. Further, there have been no changes in clinical parameters detailed in Section 4.4.

In regard to the environment, a series of studies have been carried out to demonstrate that shedding occurs infrequently, and only at early times after AvlCFl therapy. These studies are detailed in Section 4.7. In regard to the environment on the 7 West Clinical Center ward, the treated individuals will be maintained in rooms negative to the hallway (with HEPA filters for the exhaust) and under gown and glove respiratory precautions until documented to not be shedding virus (nasal, pharynx, rectal) on three separate days (see Section 5.6).

All available evidence supports the concepts that: (a) shedding of an $E1^{-}$ $E3^{-}$ adenovirus vector will be minimal in amount and duration; (b) that under the circumstances of this protocol it is unlikely that a replication competent adenovirus will be derived from a vector of this design; and (c) that a virus that poses a danger to others will not be formed or released to the environment. However, until the studies are carried out in humans with cystic fibrosis, it is not possible to prove this with absolute certainty. It is in this context that the study individuals are being asked that if they continue to shed virus (AdCFTR or an AdCFTR-derived virus), they will be asked to stay on the 7 West patient care unit in the Clinical Center until the shedding has stopped. It is recognized that the physicians caring for the study individuals can only ask the study individuals to agree to these conditions, but cannot compel them from leaving the isolation conditions and the Clinical Center at any time they choose to do so. The one exception to this is in the extraordinary unlikely, but theoretically possible, circumstance in which the physicians believed that, due to an untoward event, AdCFTR combined with another virus or other genetic information to create a new virus that was believed to be dangerous to others. In this situation NIH Clinical Center regulations allow the study individuals to be kept against his/her will for up to 72 hours. During that period, the physicians have the option to ask for a court order to keep the study individual confined until the risk to others is over. Although it is extremely unlikely for this to occur, because it is not possible to prove that it is impossible, this theoretical scenario is explicitly stated in the consent form (Section 5.13).

4.10 Is There a Risk of Malignancy Associated With the Use of This Vector?

There is no proven association of malignancy and adenovirus infection in

humans. Type 5 adenovirus is categorized in subgroup C, a subgroup that is nontumorgenic in experimental animals. Further, there is extensive clinical experience regarding the administration of live adenoviruses to humans with no reports of association with malignancy.

With this background, it is reasonable to conclude that there will be no risk for malignancy associated with this vector that will be discernable above the background risk.

4.11 Does the Genome of the Vector Integrate into the Genome of the Target Cells?

The available data suggests that adenoviruses do not integrate their genome into the genome of the target cells (Karlsson et al., 1985; Karlsson et al., 1986). It is not possible to evaluate this with freshly isolated airway epithelial cells from normals or individuals with CF (too few cells, difficulty in maintaining in culture, inability to have the cells replicate to capitalize on cloning single cells with selectable marker). The frequency of adenovirus integration is estimated to be very low (Karlsson et al., 1985; Karlsson et al., 1986).

In the context of the above, it is likely that the risk for insertional mutagenesis from AdCFTR is extremely low, far lower than that with retroviruses.

4.12 Does the Expression of CFTR Need to be Regulated?

AdCFTR and AvlCF1 are designed with a constitutive adenovirus promoter/enhancer to drive the expression of the human CFTR cDNA. In the human airway, CFTR gene expression is very low (Trapnell et al., 1991a), with somewhat higher expression in glandular serous cells than in the surface epithelial cells (J. Wilson, personal communication). The promoter has a number of putative transcriptional control regions and in vitro studies have demonstrated that inflammatory stimuli will down-regulate expression of the genes (Yoshimura et al., 1991; Chou et al., 1991; Trapnell et al., 1991b; Bargon et al., 1992).

From the safety viewpoint, since the mutations of the CFTR gene cause the airway epithelial cells to be "deficient" in CFTR function, the major safety issue regarding control of expression is to ask what are the consequences of over-expression. Three lines of evidence suggest it is not a concern.

First, in the surface epithelium of the normal lung, endogenous CFTR gene expression cannot be detected with the methods currently used. In cotton rats treated with Ad-CFTR by intratracheal administration, human CFTR gene expression is readily detectable in the surface epithelium, i.e., there is likely over-expression of the CFTR gene (see Appendix 1). This has no clinical consequences to the animals (see Appendix 1 and Section 4.4).

Second, transgenic mice constitutively expressing the human CFTR cDNA in all organs (i.e., with a promoter used by all cells) are healthy (Whitsett

et al., 1992 and personal communication).

Third, in transgenic mice in which the human CFTR cDNA is under the expression of a lung-specific promoter (human surfactant protein C), high level expression in bronchiolar and alveolar epithelial cells is observed. There were no changes in lung weight, morphology, somatic growth or reproductive capacity of these animals (Whitsett et al., 1992).

SECTION 5

HUMAN PROTOCOL

5. Human Protocol

5.1 General Design

The purpose of the protocol is to evaluate the safety and biological efficacy of administration of the recombinant adenovirus AdCFTR to the respiratory epithelium of individuals with cystic fibrosis. The overall design is that of a combined ascending dose toxicity study and biologic efficacy study with the patients serving as their own controls (prior to the administration of AdCFTR compared to after therapy, as well as during the therapy period by comparing the treated to the untreated side of the respiratory tract). At the completion of the study, the following questions will be answered:

(1) Is it safe to administer an Ela^-, (most of) Elb^-, $E3^-$ replication deficient recombinant adenovirus containing the normal human CFTR cDNA to the respiratory epithelium of individuals with CF?

(2) Will such a recombinant adenovirus transfer the exogenous normal CFTR cDNA to respiratory epithelial cells in such a fashion to permit expression of the exogenous CFTR mRNA transcripts and CFTR protein, and to correct the biologic CF phenotype (i.e., convey to the cells the ability to secrete Cl^- in response to elevations of cAMP)?

(3) How long does the biologic correction last?

(4) Is the biologic correction sufficient to correct the abnormal electrical potential difference of the airway epithelial sheet?

(5) Is there improvement in respiratory clinical parameters relevant to the disease process?

(6) Does humoral immunity develop against the recombinant vector sufficient to prevent chronic administration in the future?

5.2 Constraints in the Design of the Protocol

There are a number of clinical and safety constraints that direct the design of the protocol.

Anatomic Site of Administration of the Vector

Constraints

(1) The biologic abnormalities of CF are expressed in the nasal epithelium and in the airway epithelium. Safety considerations argue that the most conservative approach would be to first evaluate administration of the vector to the nasal epithelium. There have been prior human studies of the administration of live replication competent adenovirus to the nasal epithelium. If the vector causes acute toxicity to the

epithelium, it would be much safer to have this occur in the nose than in the bronchus. Finally, the nasal epithelium can be sampled with greater ease, safety and frequency than the bronchial epithelium.

(2) Although it is safer and easier to evaluate the vector in the nasal epithelium, the dominant clinical manifestations are between the larynx and the terminal bronchioles i.e., correction of the biologic abnormality of CF in the nasal epithelium does not offer the patient any possibility of correction of the fatal manifestation of the disease. Based on the underlying concept that there are theoretical inherent risks in administering a replication deficient recombinant adenovirus to humans, the protocol must offer possible efficacy to the patient. In this regard, it should include administration of the vector to the bronchial epithelium.

(3) There are a limited number of sites in the respiratory epithelium that can be accessed (right nostril, left nostril, right large bronchi, left large bronchi) for assessment of biologic efficacy (e.g., epithelial cells removed, potential difference measured).

(4) The epithelium can be obtained by brushing with a fine cytology brush. However, there is a limitation in the frequency of times the same site can be evaluated; at a minimum it has to be assumed that it will take 7 days to "heal" the nasal epithelium after sampling. Another consideration is the difference in accessibility of the nose and bronchi; whereas the nasal epithelium can be readily sampled, there is a limitation in the frequency of bronchoscopies that can be done to sample the bronchial epithelium in individuals with CF.

(5) Maximal information will be derived in the protocol by having as many controls as possible. Since the patients will serve as their own controls, this can be achieved in two ways: (a) by having a baseline period to assess various parameters before administration of the vector; (b) by administration of the "vehicle" used in the vector preparation to assess the effect of the vehicle on various parameters; and (c) by administration of the vector to one side (e.g. right versus left nostril, right versus left large bronchi).

(6) There are anatomic difficulties in reaching the small bronchi compared to the large airways. Further, if there are adverse effects in the bronchi, it is preferable to have these in the large airways for both accessibility and management of adverse physiologic consequences.

Conclusions Regarding Design of the Protocol

(1) Administer the vector first to the nasal epithelium, then to the large bronchi epithelium of the same individual. Administration to the nasal epithelium first will permit assessment of acute toxicity in a site with a minimal potential for airway compromise and will permit frequent assessment of biologic efficacy; administration to the large bronchi epithelium has the advantages of the possibility of clinical efficacy as well as assessment of biologic efficacy in airways, and is

safer than direct administration to small airways.

(2) Administer the vector to only one side of the nose and lung. While there undoubtedly will be some dispersion of the vector to the other side, there will be a significant concentration gradient that will permit comparison to the untreated side. Importantly, administration to only one side gives an additional measure of safety, particularly in the lung. Further, if administration to large bronchi does yield evidence of clinical efficacy, there is the chance of significant clinical improvement, since unilateral lung transplantation of individuals with CF yields marked clinical improvement.

(3) Separate the protocol into three consecutive periods: baseline, vehicle control, and AdCFTR experimental therapy.

Method of Administration of the Vector

Constraints

(1) To confine delivery to the nasal epithelium, the major constraint is volume, limited to about 0.2 ml.

(2) To the epithelium of the large bronchi, the major constraints are delivery and localization. It is possible to aerosolize adenoviruses. However, aerosolization carries with it containment problems. Direct instillation of the vector works in experimental animals and has minimal containment problems. For safety purposes, instillation should be to the right or left side (see above). Although "local" administration to a segment of large bronchus is possible in large experimental animals by using endobronchial tubes with balloons and catheters, this would be very difficult in individuals with CF (e.g., risk of general anesthesia, achieving air exchange distal to the balloon without the risk of atelectasis, the balloons may damage the epithelium, higher risk for infection, etc.)

(3) Since the disease is limited to the airway epithelium from the larynx to the small bronchioles, it would be preferable to limit, as much as possible, the delivery of the vector to the airways and not to the alveolar epithelium. There is data that with direct instillation of 20 ml of fluid to the human large airways, most of the fluid will stay in the airways and not reach the alveoli (Smith et al., 1988).

Conclusions Regarding Method of Administration of the Vector

(1) Direct instillation to the right or left nostril, with limitation of volume to 0.2 ml.

(2) Direct instillation to the right or left mainstem bronchus via a bronchoscope, with limitation of volume to 20 ml.

Immunity

Constraints

(1) Almost all adults have evidence of anti-adenovirus antibodies in blood (Strauss, 1984) and studies in cotton rats have shown that a one time administration of AdCFTR to the respiratory tract induces anti-AdCFTR antibodies in blood within 1 week (Section 4.5). Vaccine-related trials in non-human primates and humans suggest that administration of adenovirus vectors containing an exogenous gene to the respiratory tract does not elicit significant humoral immunity to the exogenous gene product, but there is anti-adenovirus humoral immunity. Thus, it is prudent to assume that the initial respiratory administration of AdCFTR (nasal or bronchial) may elicit anti-AdCFTR antibodies in blood and/or lung. If so, there are three possible consequences: (a) the antibodies are irrelevant and there will be no consequences; (b) the antibodies will modulate an inflammatory process in the airways upon re-challenge with AdCFTR; and (3) the antibodies will be neutralizing, thus preventing the vector from reaching the target cells upon re-challenge.

Conclusions Regarding Design of the Protocol

(1) First administer the vector to the nasal epithelium. If after 24 hr, there is no sign of acute toxicity, administer the vector to the large bronchi of the same individual. The span of 24 hr will permit assessment of acute toxicity, but will not be sufficient for the development of an initial or anamnestic response to the adenovirus vector.

(2) This design will permit evaluation of the anti-vector humoral immunity in blood and lung as a function of time, permitting rational design of future protocols.

Dose

Constraints

(1) Because AdCFTR is a new "drug", it will be necessary to evaluate possible toxicity with ascending doses. There are limitations, however, in how this can be achieved. Because of the constraint of possible immune reactions, the toxicity evaluation cannot be achieved by ascending dosages in the same individual. Further, to offer a possibility of benefit to the individual the starting dose cannot be too low.

(2) There is a limitation in volume that can be administered to the nostril compared to the bronchi, but the volume will spread out over the respective epithelial sheets. Thus, if the same titer is

administered in the two sites, comparable dosage (i.e., plaque forming units (pfu)/cell) will be given to the epithelial cells of all sites.

(3) The maximum titer of adenovirus that can be repeatedly achieved in production is $1x10^{11}$ to $5x10^{11}$ pfu/ml. This, together with the limitation of volume, puts an upper limit on dose.

Conclusions Regarding Design of the Protocol

(1) Use different patients for ascending doses, with n=2 at each dose. Continue to add n=2 patient groups until the maximum dose is reached or toxicity is observed.

(2) Start with a total dose in the range that has been used to assess the responses of humans to replication competent adenovirus. For a 60 kg male (CF individuals tend to have less body mass than normals), start with titers of 10^6 pfu/ml (total dose to the lung with 20 ml volume will be $2x10^7$ pfu/60 kg = $3.3x10^5$ pfu/kg) for the first two individuals and increase in groups of n=2 until the highest dose (20 ml x 10^{11} pfu/ml-60 kg = $3.3x10^{10}$ pfu/kg) is reached.

Study Population

Constraints

(1) It can be argued that rather than starting with individuals with CF, it would be much easier to determine if there are any adverse effects from the Ad vector or the CFTR cDNA if the initial study population were normal volunteers. First, regarding the Ad vectors, this would not be different from evaluating live adenovirus or adenovirus vaccines in normal humans, studies which have been extensively evaluated and reported in the literature (see Table 5.2-A). Second, the Ad vector to be utilized in this study, AdCFTR, expresses a normal CFTR cDNA. Potential adverse effects generated in response to expression of the CFTR protein must be balanced against potential benefits to individuals. The potential benefit in CF individuals will be amelioration or cure of the respiratory manifestations of the disease. In contrast, while there is no experimental evidence to argue that AdCFTR, in the same dosages, would be harmful to normals, no benefit in normals would be expected. Within the CF population, individuals with at least one allele known to produce the CFTR protein will be selected, as these individuals will be less likely to respond immunologically to expression of the exogenous gene.

(2) As it is a goal of the protocol to be able to demonstrate conversion of airway epithelial cells from an abnormal CF phenotype to a normal phenotype, it is mandatory that the CF genotype is known.

Conclusions Regarding Design of the Protocol

(1) The subjects will have CF as identified by conventional clinical

Table 5.2-A

Representative Literature Regarding Administration of Replication Competent Adenovirus to Humans by the Respiratory or Oral Routes

Route/Reference	n	Virus	Dose	Purpose	Clinical Sequelae	Shedding[1]	Antibodies[2]
Respiratory							
Ginsberg et al., 1955[3] nasal	24	Ad4	unknown	etiology of respiratory disease	upper respiratory symptoms[4]	not tested	75% +
Roden et al., 1956 nasal	11	Ad1	10^4 pfu	induction of respiratory disease	mild respiratory symptoms in 2/11 at 2-7 d[4]	throat 35% +	40% +
Bell et al., 1956 nasal inhaled	574	Ad1-6	unknown	induction of respiratory disease	none	not tested	44-69% +
Couch et al., 1966 inhaled	15	Ad4	10^1-10^3 pfu	role of lower respiratory inoculation	mild respiratory symptoms at 6-13 d pneumonia in 2[4]	anal 100% + at 3-20 d throat 100% + at 5-20 d	100% +
Smith et al., 1970 nasal	58	Ad4	10^3-10^6 pfu	prevention of disease with intermuscular vaccine	none	nasal 70% + at 2-12 d	100% +
Oral[5]							
Couch et al., 1963 enteric	15	Ad4,7	10^3-10^6 pfu	oral vaccine development	conjunctivitis in 1[4]	rectal 100% + at 2-24 d	>90% +
Chanock et al., 1966 enteric	46	Ad4	10^3-10^6 pfu	oral vaccine dose and safety	none	anal 95% + at 21 d throat -	>90% +
Edmondson et al., 1966 enteric	134	Ad4	10^5-10^6 pfu	oral vaccine efficacy	none	anal 95% + throat -	>90% +
Gutekunst et al., 1967 enteric	360	Ad4	10^6-10^7 pfu	oral vaccine dose and safety	none	anal 100% + at 8 d throat -	100% +
Rosenbaum et al., 1968 enteric	65	Ad3,4,7	10^6 pfu	efficacy during epidemic Ad	none	rectal 95% + at 21 d throat 30% + at 28 d	90% +
Stanley and Jackson, 1969 enteric	39	Ad4	10^5-10^6 pfu	spread of Ad vaccine in married couples	none	rectal 50% + at 6 wks oral 10% + at 6 wks	50-100% +
Top et al., 1971c enteric	55	Ad4,7	10^4-10^6 pfu	oral vaccine safety antigenicity	none	fecal 80-90% + at 21 d	95% +
Top et al., 1971a enteric	88	Ad4,7	10^4 pfu	oral vaccine efficacy	none	not tested	65-78% +
Scott et al., 1972 enteric	15	Ad21	10^4 pfu	antibodies nasal vs enteric	none	rectal 90% +	90% +

[1]Shedding + = Ad detected in sample after ad administration
[2]Antibody + = 4-fold rise in neutralizing ad between pre and post treatment sera in naive individuals
[3]Analysis of specimens from previous report: The Commission on Acute Respiratory Diseases, 1947a and ibid, 1947b
[4]Significant clinical sequela occurred in only individuals who had no serum anti-Ad antibodies pretreatment
[5]Additional references regarding enteric Ad trials include: Pierce et al., 1966; Peckinbaugh et al., 1968; Pierce et al., 1968; Griffin et al., 1970; Top et al., 1971b; Rosenbaum et al., 1975

criteria, including a positive sweat chloride test.

(2) The CF genotype will be identified.

(3) The CF genotype will be either "delta F508" homozygotes or the "delta F508" mutation on one allele and on the second allele, a null mutation known not to produce CFTR mRNA (i.e., a delta F508-null compound heterozygote at the mRNA level). The purpose of this genotype restriction is for technical convenience based on the goal of demonstrating that the administration of AdCFTR in vivo will change the genotype of the respiratory epithelium at the mRNA level (using sequence analysis and PCR with specific probes; see Section 5.5.3.1). With this genotype restriction, the sequence and specific probe analyses will be definitive pre-therapy and after correction.

Ability to Give Informed Consent

Constraints

(1) The protocol is complex, with major implications for the study population in regards to risk/benefit considerations, and decisions of the study population in regards to the implications of personal freedoms imposed by the AdCFTR experiment treatment period (see sections 5.6.2 - 5.6.4).

Constraints Regarding Design of The Protocol

(1) All patients must be 21 years or older.

Protection from Germ Line Gene Transfer

Constraints

(1) The target for gene therapy for this protocol is the respiratory epithelium of the airways. Studies are ongoing in cotton rats and non-human primates to demonstrate that there is no transfer to other organs (including gonads) when the vector is administered by the respiratory route. However, as it is not possible to develop absolute evidence that germ line transfer will not occur when a vector is administered in vivo by the respiratory route, the protocol should be carried out in individuals that have definitive evidence of being incapable of bearing offspring. Relevant to this consideration is that almost all males with CF are sterile (Stern et al., 1982; Boat et al., 1989).

Conclusions Regarding Design of the Protocol

(1) As inclusion criteria to enter the vehicle control period (see section 5.6.3) and AdCFTR experimental treatment period (see 5.6.4), studies in the baseline period (see 5.6.3) must document sterility in males

(semen analysis), and in females (the absence of a uterus and/or ovaries).

Necessity to Frequently Obtain Biologic Specimens From the Airways

Constraint

(1) To demonstrate biologic efficacy in the airways, it is necessary to frequently obtain biologic specimens by fiberoptic bronchoscopy, a procedure that requires informed consent and is associated with some morbidity (fevers, occasional infections) in individuals with CF. The procedure requires local anesthesia and is associated with some discomfort. In addition, the subject has to have lung function adequate to permit frequent bronchoscopy, and airways large enough to permit use of an adult bronchoscope (because of the diameter of the channels used for sampling).

Conclusion Regarding Design of the Protocol

(1) All patients must have demonstrated ability to undergo the bronchoscopy and sampling procedures without difficulty.

Recombination, Complementation and Environmental Concerns

Constraints

(1) AdCFTR is designed to be replication deficient in that it is Ela^- and (most of) Elb^- and it does not replicate in freshly isolated human airway epithelial cells at levels that are detectable. However, addition to AdCFTR of the appropriate genetic information, either by recombination or by complementation in <u>trans</u>, may result in the production of more AdCFTR (by complementation) or a replication competent virus (by recombination).

(2) AdCFTR is an infectious agent, albeit designed to be replication incompetent. There is a theoretical risk to the environment for release of AdCFTR and/or a recombinant virus, mediated either from genetic information in the respiratory epithelium or from an exogenous viral source.

Conclusions Regarding Design of the Protocol

(1) Evaluate patients in the baseline period to ensure that they do not harbor an infectious virus relevant to the respiratory tract.

(2) Evaluate the respiratory epithelium of patients prior to inclusion to insure that they do not harbor Ad5 DNA sequences for Ela, a region deleted in AdCFTR.

(3) Prior to inclusion in the protocol, recover airway epithelial cells from individuals with CF, infect with AdCFTR and demonstrate that the

DNA from AdCFTR will not replicate.

(4) Include in the protocol only individuals with demonstrated anti-adenovirus type 5 antibodies in blood. While this will not ensure absolute protection should recombination to a replication competent adenovirus occur, the presence of anti-Ad5 antibodies in blood is evidence of prior exposure to Ad5 (or a cross-reacting Ad serotype), and likely provides some additional measure of safety compared to an individual that has no anti-Ad5 antibodies (see Table 5.2-A).

(5) After inclusion in the protocol, the patients will be clinically isolated for 10 days prior to being treated with the vector (i.e., to prevent exogenous viral infection) and will be clinically evaluated prior to administration of the vector to ensure there is no intercurrent viral illness. Isolation will entail restriction to the individual's hospital room on the 7W patient care unit as much as possible. Visitors to the room must be 15 years or older. All visitors and hospital personnel will wear gown, mask, and gloves. When the individual leaves the room, he/she will wear a mask and gloves and be accompanied by hospital medical/nursing staff.

(6) After administration of the vector, the patient will be kept in isolation until demonstrated not to be shedding the vector or replication competent adenovirus from the nose, pharynx or rectum on 3 separate days.

(7) The patient rooms to be used on the 7W ward will be converted so as to be pressure negative relative to the corridor, and have air leaving the rooms appropriately filtered, thus minimizing risk of release to the environment.

(8) Patients will not be included who are taking corticosteroid or other known immunosuppressive medications.

(9) In regards to the environment, it is recognized that following the administration of AdCFTR should the study individual wish to leave isolation conditions and/or the Clinical Center prior to the demonstration of a lack of shedding of AdCFTR or an AdCFTR-derived virus, the physicians caring for the study individual cannot compel that individual to remain. The one exception to this is the extraordinarily unlikely, but theoretically possible, circumstance where the physicians caring for the study individual believe that due to an untoward event, AdCFTR combined with another virus or other genetic information to create a new virus that was believed to be dangerous to others. In this event, the NIH Clinical Center regulations allow the physicians to keep the study individual against their will for up to 72 hours. During that period they would have the option to ask for a court order to keep the study individual confined until the risk to others is over. The risks to the environment and this general concept are discussed in detail in section 4.9. This theoretical scenario will be explicitly explained to the study individual and is in the informed consent document.

5.3 Baseline Adenovirus-related Data from Individuals with CF Relevant to Administration of a Replication Deficient Recombinant Adenovirus

During the baseline period of the protocol, baseline parameters relevant to the adenovirus vector will be evaluated, including baseline immunity against type 5 adenoviruses, the presence of infectious viruses in the respiratory tract, the baseline inflammation in the respiratory tract, and the presence of type 5 adenovirus Ela sequences in the respiratory tract.

5.3.1 Anti-Adenovirus Antibodies in Blood and Lung of Individuals with CF

Consistent with data demonstrating that most adults have some antibodies against adenovirus type 5 in serum (Strauss, 1984), evaluation of the individuals with cystic fibrosis entering the protocol will likely demonstrate circulating antibodies against adenovirus. It is also possible that anti-type 5 adenovirus antibodies will be detected in the respiratory epithelial lining fluid (ELF) of the individuals with cystic fibrosis. However, even if present, it is unlikely that anti-adenovirus antibodies on the respiratory epithelial surface will be active, as: (1) almost all individuals with CF have active neutrophil elastase in CF respiratory ELF; (2) neutrophil elastase will cleave immunoglobulins; and (3) there is in vivo evidence that immunoglobulins in ELF from individuals with CF are cleaved (Fick et al., 1984). Despite the unlikely possibility that such antibodies could survive in CF ELF, studies will be done during the baseline period to determine whether anti-type 5 adenovirus antibodies are present in the serum and lung of the individuals with CF, and if detectable, whether such antibodies are neutralizing.

5.3.2 Infectious Viruses in the Respiratory Tract of Individuals with CF

To minimize the risks for recombination or complementation of AdCFTR with an intercurrent respiratory virus infection, all individuals will be screened during the baseline period for infection with respiratory viruses in the respiratory tract. The absence of such an infection will be an inclusion criterion (see Section 5.4.1). This criterion was established based on screening of individuals with CF for such viruses.

Individuals with CF (n=17 evaluation for all viruses, all sites; n=21 total) were screened in the respiratory tract (nasal brushing, bronchial brushing, and bronchoalveolar lavage) for the presence of: adenovirus (all serotypes except 40, 41), cytomegalovirus, varicella zoster virus, herpes simplex viruses I and II, influenza A and B, parainfluenza 1, 2 and 3, and respiratory syncytial virus. Control groups included normal non-smokers, normal smokers, and individuals with alpha 1-antitrypsin deficiency.

All cultures in the control groups were negative for all sites and all viruses. All CF individuals were negative for adenovirus, cytomegalovirus, varicella zoster virus, influenza A and B, parainfluenza 1, 2 and 3, and respiratory syncytial virus at all sites. Two of 17 (12%) of individuals with CF had culture evidence of herpes simplex virus [one individual in bronchial epithelium and respiratory epithelial inflammatory cells (bronchoalveolar lavage), one individual in lavage only, both were negative in the nasal epithelium]. See Table 4.9-D for details.

5.3.3 Baseline Inflammation in Respiratory Epithelium of Individuals with CF

The type and number of inflammatory cells in nasal epithelium and bronchial epithelium and respiratory epithelial lining fluid will be used as safety and efficacy parameters, with increases in numbers of inflammatory cells suggesting adverse events and decreasing numbers suggesting efficacy (see below for caveats regarding these parameters). The extent of inflammation in each site in a group of individuals with CF as compared to non-smoking normals is detailed in Section 4.4.

In the nasal epithelium of individuals with CF there is more inflammation compared to normals, but it is relatively mild. In contrast, in the bronchial epithelium in CF there is an intense, neutrophil dominated inflammatory process with a more than 30-fold increase in the numbers of neutrophils present among the epithelial cells recovered by bronchial brush compared to normals (Table 4.4-A). The extent of the inflammation on the epithelial surface of the CF lung is further documented by quantification of the numbers of inflammatory cells recovered by bronchoalveolar lavage: in CF more than 4-fold more inflammatory cells are recovered and there is a 300-fold increase in the number of neutrophils present (Table 4.4-A).

These observations highlight the extensive epithelial-based inflammation in CF which are consistent with morphologic observations of the CF lung in specimens obtained at autopsy or at lung transplantation (Bedrossian et al, 1976). The methods used to quantify these parameters (nasal brush, bronchial brush and bronchoalveolar lavage), however, are not sufficiently well established to permit their use as definitive criteria for establishing the presence or absence of adverse effects or efficacy. Therefore, these parameters will be collected throughout the protocol, but will not be used as criteria for safety and efficacy unless the observations are dramatic and repeated.

5.3.4 DNA Containing Ela Sequences in Nasal Epithelial Cells, Bronchial Epithelial Cells, Lung Inflammatory Cells, Blood Lymphocytes and Blood Neutrophils of Individuals with CF

Based on the knowledge that AdCFTR could theoretically recombine with exogenous adenovirus sequences, or that E1 sequences could provide sufficient information, in <u>trans</u>, to complement the E1 sequences deleted from AdCFTR, to minimize the risks of recombination or complementation, all individuals entered into the baseline period of the protocol will be assessed for the presence of adenovirus Ela sequences in the airway epithelium. Only those negative for the presence of Ela sequences will be accepted to move to the vehicle control period (see inclusion criteria, section 5.4). These criteria were developed based on assessment of respiratory epithelium of individuals with CF for the presence of Ela sequences (see Section 4.9, Table 4.9-C).

The data demonstrate that Ela DNA sequences are found in the epithelium of the respiratory tract of 10% of individuals with CF. Of those individuals that are Ela^+, there were an average of 87 $\pm$ 26 copies of Ela/10^3 epithelial cells. This is no different from normal individuals (see Table 4.9-C for details).

5.4 Patient Eligibility and Selection

Based on the constraints inherent in the disease and the vector system, and the need to maximize safety and gain access to biologic specimens to demonstrate biologic efficacy, the following are the inclusion and exclusion criteria for the study population. Patients with cystic fibrosis meeting these criteria will be chosen from among patients participating in ongoing Pulmonary Branch, NHLBI studies and those referred to the Pulmonary Branch.

5.4.1 Inclusion criteria

Males or females 21 years or older

Cystic fibrosis defined by routine clinical criteria, a positive sweat chloride test, a genotype of the "delta F508" mutation on both CFTR alleles or a compound heterozygote of the type "delta F508-null", with the null mutation of a type known not to produce CFTR mRNA (see below).

Documented sterility in males (semen analysis) and females (documentation of absence of uterus and/or ovaries)

Lung disease typical of CF, with FEV1 > 1.2 L/sec

Shwachman score 40-70 (mild to moderate disease) (Shwachman and Kulczycki, 1958). If there is gastrointestinal or liver disease present, it must be mild and stable with appropriate therapy

If diabetes mellitus is present, it must be stable, with no history of diabetic ketoacidosis, hyperosmolar coma or hypoglycemia requiring hospitalization within the previous three years

Demonstrated ability in the baseline period to undergo nasal brushing once weekly without complications

Demonstrated ability in the baseline period to undergo fiberoptic bronchoscopy with brushing of the large airways and bronchoalveolar lavage once monthly without complications

Reproducible respiratory epithelial potential difference (to subcutaneous tissues) of > -40 mV and/or airway epithelium unable to secrete Cl^- in a normal fashion in response to cAMP

Airway epithelial CFTR mRNA demonstration of CFTR mutations of the type "delta F508 homozygote" or a compound heterozygote "delta F508-null"

No detectable CFTR protein in airway epithelium (using methods described in Section 3.1.2)

Not taking experimental medications relevant to lung disease of CF for at least 4 weeks prior to entry into study, including recombinant human DNase, anti-proteases such as alpha 1-antitrypsin or recombinant secretory leukoprotease inhibitor, or amiloride

Ability to stay at the Clinical Center, NIH for a minimum of 4 months, and to return to the Clinical Center once monthly for 10 months and at 6 month intervals for one year, and then once yearly thereafter

During the AdCFTR experimental treatment period, ability and agreement to remain in the Clinical Center, 7 West ward under appropriate isolation conditions, until blood, urine, nasal, pharynx and rectal cultures are negative on 3 separate days. Visitors will be limited to individuals 15 years or older.

Evidence in blood of antibodies against adenovirus type 5

Assessment of airway epithelium demonstrating no evidence of adenovirus type 5 Ela sequences (see Section 4.9)

Culture of bronchial brush, nasal brush and bronchoalveolar lavage samples demonstrating no evidence of adenovirus, cytomegalovirus, varicella-zoster, herpes simplex I and II, influenza viruses A and B, parainfluenza viruses 1, 2 and 3, or respiratory syncytial virus

No evidence of replication of AdCFTR DNA upon in vitro infection of the patient's respiratory epithelium with AdCFTR (see Section 4.1)

No anatomic anomalies that would prohibit instillation of the vector and recovery of respiratory epithelium as dictated by the protocol

No history of allergy to glycerol or medications used in the bronchoscopy procedure

No history of documented adenovirus-mediated tracheobronchitis and/or pneumonia

Capable of providing informed consent

5.4.2 Exclusion criteria

Unable to meet the inclusion criteria.

In the 3 weeks prior to beginning the AdCFTR experimental treatment period, no evidence of active respiratory tract infection including evidence of fever, rhinitis, pharyngitis, conjunctivitis, bronchitis or changes in sputum requiring antibiotics or a change in antibiotics. If there is evidence of active respiratory tract infection, the individual will be discharged (after the respiratory tract infection stabilizes), and the individual reevaluated 4 weeks later. If all of the inclusion criteria are met, the individual will enter the AdCFTR experimental treatment period.

Evidence of cardiac, renal, central nervous system, major psychiatric disorders, musculoskeletal disease or immunodeficiency disease (including evidence of human immunodeficiency virus infection).

5.5 Safety and Efficacy Parameters

The patients will be monitored with a variety of safety and efficacy parameters during the baseline, vehicle control and AdCFTR experimental therapy period. Some of the parameters will be used for both safety and efficacy assessment.

5.5.1 Definitions

The terms listed on the left will be used in the subsequent sections (5.5 Safety and efficacy parameters, 5.6 Clinical protocol); The definitions of each term (i.e., the group of assays each term include), are listed on the right.

General assessment	- history, physical exam, vital signs (blood pressure, pulse, respiratory rate, temperature), weight
Dyspnea index	- subjective index defined by a questionnaire (Stoller et al., 1986)
General blood	
CBC, ESR	- complete blood count, differential, platelets, erythrocyte sedimentation rate
Clotting	- prothrombin time, partial thromboplastin time, fibrinogen level
Chemistry	- sodium, potassium, chloride, total CO_2, blood urea nitrogen, creatinine, alkaline phosphatase, alanine amino transferase, aspartate amino transferase, bilirubin (total), bilirubin (direct), total protein, albumin, calcium, magnesium, phosphate, uric acid, glucose, creatine phosphokinase, lactic dehydrogenase, cholesterol, triglycerides.
Other	- serum sample frozen for future use
General immunity	- anti-nuclear antibodies, rheumatoid factor, complement level (CH50), complement components 3 and 4, immunoglobulin G, immunoglobulin M, immunoglobulin A, immunoglobulin E
Culture	- routine blood culture
HIV	- one pre-study test only
Urine	
Routine	- appearance, specific gravity, pH, protein, glucose, ketones, bilirubin, hemoglobin, number and type of cells, characterization of sediment
Culture	- routine urine culture

24 hr	- creatinine clearance, total protein
Sterility evaluation	- semen analysis (males); ultrasonographic evaluation of pelvis (woman)
EKG	- electrocardiogram
Roentgenographic	
Chest X-ray	- posterior-anterior, lateral (Brasfield semiquantitative scoring) (Brasfield et al., 1980)
Chest CT	- chest computed tomography (Bhalla semiquantitative scoring) (Bhalla et al., 1991)
Scintigraphic (V/Q)	- ^{133}Xe ventilation scan ^{99m}Tc-macroaggregated albumin perfusion scan
Lung function	
Spirometry	- forced vital capacity, forced expiratory volume in 1 sec, forced expiratory flow 25-75%
He dilution, DLCO	Helium dilution (total lung capacity, functional residual capacity, residual volume), diffusing capacity (single breath)
Body box	- body plethysmography (total lung capacity, airway resistance)
ABG	- arterial blood gases (pO_2, pCO_2, pH, saturation)
Sputum	
Volume	- 17 hr, 7 am to 12 pm
Culture	- routine and quantitative bacterial culture
Bronchoalveolar lavage	- cell number, type, culture (routine and quantitative bacterial culture), volume of epithelial lining fluid (urea method), mediators (neutrophil elastase, interleukin 8, tumor necrosis factor)
Nasal brush	- cell number, type, culture (routine and quantitative bacterial culture, virus culture), biologic efficacy [expression of normal CFTR genotype at mRNA level (sequencing and PCR with specific probes); level of total CFTR mRNA (quantitative PCR, Northern, <u>in situ</u>); secretion of Cl^- in response to intracellular elevations of cAMP ($^{36}Cl^-$

efflux, SPQ dye); expression of CFTR protein (immunohistochemistry, immunoprecipitation with kinase reaction, ^{35}S-methionine metabolic labeling and immunoprecipitation)]

Bronchial brush - cell number, type, culture (routine and quantitative bacterial culture, virus culture), biologic efficacy (same as for nasal brush)

Potential difference - nasal, bronchial (if bronchoscopy performed)

Vector-related

Anti-Ad antibodies - anti-AdCFTR, anti-Ad5 (serum and lavage fluid if bronchoscopy performed)

Adenovirus culture - blood, rectal, urine, pharyngeal, nasal brush (if performed), bronchial brush (if performed)

Adenovirus DNA - specific DNA sequences from AdCFTR (CFTR cDNA) and from wild type Ad5 (Ela) - evaluated in blood, nasal brush (if performed), bronchial brush (if performed)

Serum TNF - levels of tumor necrosis factor

5.5.2 Safety Parameters

The patients will be monitored with a variety of safety parameters during the baseline, vehicle control, and AdCFTR experimental treatment periods. The safety parameters are divided into two categories, "general clinical parameters" to monitor the individual's overall status as well as specific organ systems, and "vector-specific parameters" specifically relevant to the administration of a replication deficient recombinant adenovirus. The definitions for each group of parameters are in Section 5.5.1. The times of assessment of each parameter are detailed in Section 5.6.

5.5.2.1 General Clinical Safety Parameters

The general clinical safety parameters include (see Section 5.6 for specific times of evaluation):

General assessment
Dyspnea index
General blood (CBC, ESR, clotting, chemistry, other, general immunity, culture, HIV)
Urine (routine, culture, 24 hr)
Sterility evaluation
EKG
Roentgenographic (chest X-ray, chest CT)
Scintigraphic (V/Q)

Lung function (spirometry, He dilution, DLCO, body box, ABG)
Sputum (volume, culture)
Bronchoalveolar lavage (cell number, type, culture, volume)
Nasal brush (cell number, type, culture)
Bronchial brush (cell number, type, culture)

The total maximum radiation exposure for roentgenographic and scintigraphic parameters for the first year of the protocol is approximately 4 rads. This amount is judged safe by NIH guidelines (less than 5 rads per year allowable).

5.5.2.2 Vector-related safety parameters

The safety parameters directly related to the AdCFTR vector include (see Section 5.6 for specific times of evaluation):

Anti-Ad antibodies (serum, lavage)
Adenovirus culture (nasal brush, bronchial brush, pharynx, blood, rectal, urine)
Adenovirus DNA - (nasal brush, bronchial brush, blood)

5.5.3 Efficacy Parameters

Evaluation of the efficacy of a recombinant adenovirus containing the human CFTR cDNA to treat the respiratory manifestations of cystic fibrosis will be based on biologic and clinical parameters.

5.5.3.1 Biologic Efficacy Parameters

The focus of these parameters is to demonstrate that the recombinant vector will compensate for the endogenous abnormal CFTR genes to provide normal CFTR gene-related expression to the airway epithelial cells. Most of these parameters will be evaluated in nasal and bronchial airway epithelial cells obtained by brushing the epithelium (see description of methods in sections 3, 4 and references Chu et al., 1991; Trapnell et al., 1991a). The epithelial cells will be recovered periodically during the baseline period (see section 5.6.2), the vehicle control period (section 5.6.3) and during the AdCFTR experimental treatment period (see section 5.6.4). The times of assessment of each parameter are detailed in section 5.6. The parameters to be evaluated (see sections 3.1, 3.2 for details as to methodology), include:

Expression of normal CFTR genotype at the mRNA level (sequence, PCR with specific probes)

Level of total CFTR mRNA (quantitative PCR, Northern, <u>in situ</u> hybridization analysis)

Secretion of Cl^- in response to intracellular elevation of cAMP ($^{36}Cl^-$ efflux, SPQ dye)

Expression of CFTR protein (immunohistochemistry, immunoprecipitation and phosphorylation with kinase reaction, ^{35}S-methionine metabolic labeling

and immunoprecipitation)

In addition to the evaluation of biologic correction in airway epithelial cells removed by brushing, the CFTR-related function of the intact respiratory epithelial sheet will be evaluated by quantifying the potential difference between the surface of the airway epithelium and the subcutaneous tissues. In normal individuals the potential difference is -25 ± 1 mV, whereas in CF it is > -40 mV (Knowles et al., 1981).

To measure the nasal electrical potential an intravenous catheter is placed subcutaneously in the forearm and connected to an infusion of Ringers solution. Less than 1 ml of Ringers solution is infused to flush the catheter and establish continuity of the subcutaneous space and the infused solution. A second catheter, the "exploring catheter", is filled and constantly perfused with Ringers solution. A Y-connection is present in both infusion lines with a silver chloride electrode in one of the limbs of each. Both electrodes are connected to a battery operated, high impedance volt meter, which is connected to a chart recorder. The exploring catheter is placed on the surface of epithelia in various locations within the nasal cavity and the electrical potential difference between the mucosal surface and the interstitial space is measured. Visualization is achieved with a headlight and nasal speculum. To measure tracheal and bronchial potential, the equipment is set up in the same manner as for measuring nasal potential, the only difference being that the infusion for the exploring catheter is connected to a length of polythene tubing that can be placed through the suction channel of the bronchoscope and then rested on the tracheal or bronchial surface.

Increased serum levels of tumor necrosis factor, have been correlated with exacerbations of cystic fibrosis (Suter et al., 1989a); this parameter will also be followed.

The biologic efficacy parameters relating to the respiratory epithelium are central to the goals of this protocol. In this context, and in recognition that not all parameters can be measured at each time point because of limitations in biologic materials, inability for a bronchoscopy to be carried out appropriately for clinical and/or technical reasons and variability in the biologic parameters, the following categorizes the respiratory epithelial-related biologic parameters into primary and secondary efficacy parameters. All efforts will be focused on obtaining, at a minimum, the primary efficacy parameters. The primary parameters will include: expression of normal CFTR genotype at the mRNA level (sequence and PCR with specific probes) in nasal and bronchial epithelium, expression of CFTR protein (immunohistochemistry) in nasal and bronchial epithelium, and measurement of potential difference across the nasal epithelium. The secondary parameters will include: level of total CFTR mRNA (quantitative PCR, Northern, <u>in situ</u> hybridization) in nasal and bronchial epithelium, secretion of Cl^- in response to intracellular elevation of cAMP ($^{36}Cl^-$ efflux, SPQ dye) in nasal and bronchial epithelium, and expression of CFTR protein (immunoprecipitation and phosphorylation with kinase, ^{35}S-methionine metabolic labeling and immunoprecipitation) in nasal and bronchial epithelium.

5.5.3.2 Clinical Efficacy Parameters

These parameters will assess whether administration of the recombinant vector is associated with efficacy directly and clinically relevant to the patient. Some of these parameters are also used to monitor safety (see section 5.5.2). The clinical efficacy parameters include:

General clinical

Weight
Dyspnea index
Incidence of airway infections requiring antibiotic therapy
Number and length of hospitalizations (other than as dictated by the protocol) for respiratory illness

Roentgenographic

Chest X-ray
Chest CT scan

Scintigraphic

V/Q scan

Lung function

Spirometry, He dilution, DLCO, body box, ABG

Sputum

Volume, culture

5.6 Clinical Protocol

5.6.1 Overview

The clinical study will include 10 individuals with cystic fibrosis, grouped into 5 groups based on the titer of vector that will be administered. In order to maintain a constant relative dose to the epithelium in the nose and airways, the volumes (0.2 ml to nose, 20 ml to large bronchus) are based on the estimates of surface area that will be exposed to the vector, 0.01 m^2 in the nose, 1.0 m^2 in the bronchi, a 100-fold difference):

group 1:	n=2,	titer of vector 10^6
group 2:	n=2,	titer of vector 10^8
group 3:	n=2,	titer of vector 10^9
group 4:	n=2,	titer of vector 10^{10}
group 5:	n=2,	titer of vector 10^{11}

Each individual will serve as their own control, by comparing parameters in the initial baseline period and the vehicle control period to the AdCFTR experimental treatment period, as well as in the experimental treatment period by comparison of the untreated side (nose and lung) to the treated side (nose

and lung).

The study individuals may go through the baseline period and vehicle control period at any time prior to entering the AdCFTR experimental treatment period, but if the time between the end of the vehicle control period and beginning of the treatment period is greater than 1 month, an additional baseline evaluation will be carried out 10 days prior to starting the treatment.

In the description of baseline, vehicle control, and AdCFTR experiment treatment periods that follows, there is an accompanying time chart that details the sites of administration of the vehicle or AdCFTR (for the vehicle control period and AdCFTR experimental treatment period, respectively), the time of sampling of the nasal and large bronchus epithelia, and the time for assessment of various safety and efficacy parameters. A list of the parameters within each category and the abbreviations used can be found in section 5.5.

5.6.2 Baseline Period

Prior to start of the baseline period, there will be an initial evaluation to examine safety and efficacy parameters. This data, plus the data gathered throughout the baseline period will determine if the individual is eligible to continue in the protocol (see Inclusion and Exclusion criteria, sections 5.4.1, 5.4.2). If eligible for the remainder of the protocol, the individual will enter the vehicle control period within 1 month following the completion of the baseline period. Following the initial evaluation, the baseline period lasts 30 days (see Figure 5.6.2-A). The baseline period will be used to evaluate the reproducibility of various parameters as well as the ability of the individual to meet the inclusion/exclusion criteria.

The specific times for assessment of each parameter may be found in Figure 5.6.2-A.

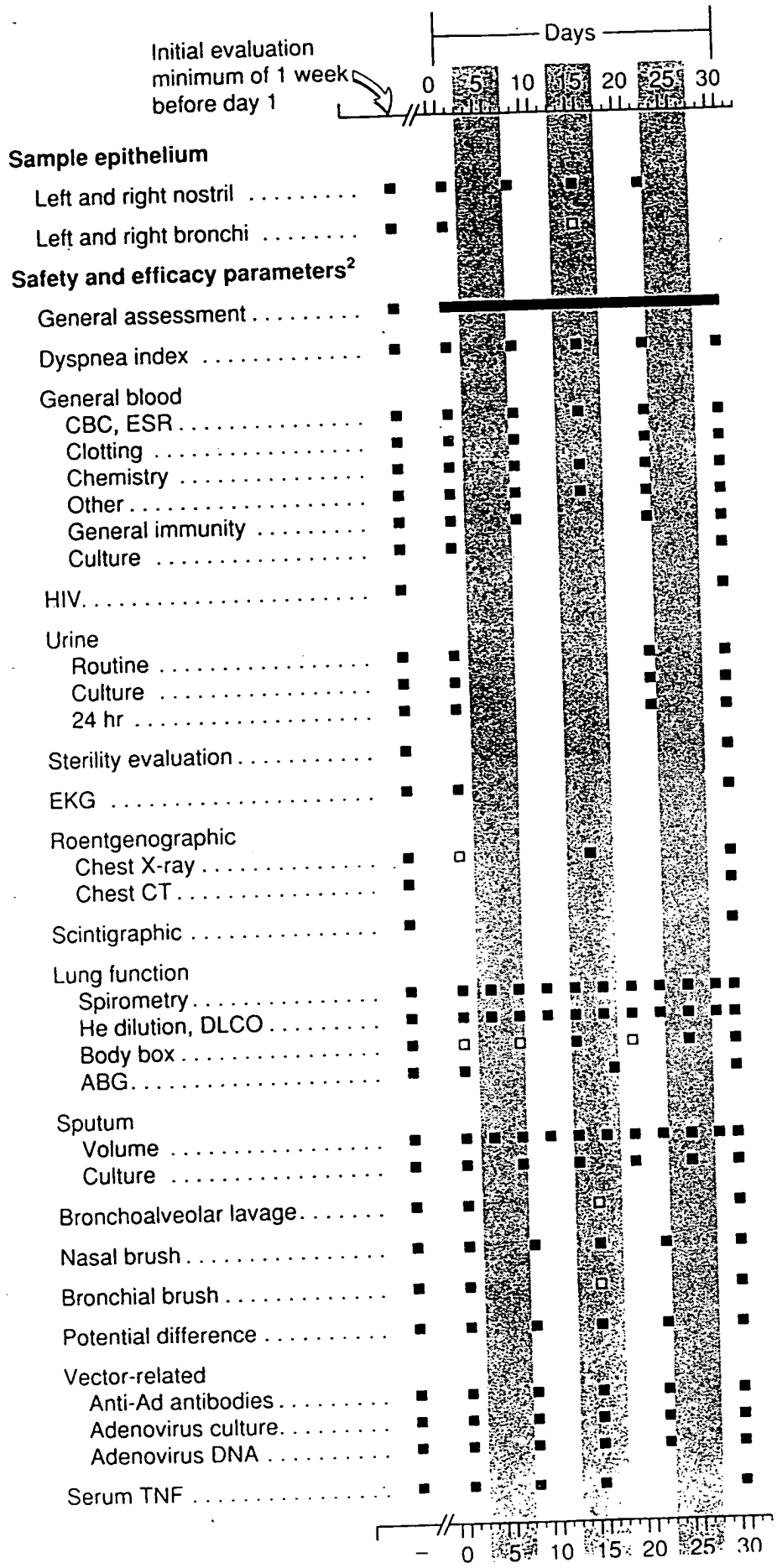
5.6.3 Vehicle Control Period

The vehicle control period will be to evaluate the same safety and efficacy parameters evaluated in the baseline period, except that the vehicle to be used in the AdCFTR preparation (10 mM Tris-Cl, pH 7.4, 1 mM $MgCl_2$, 3.3% glycerol) will be administered to the nostril and bronchi in a fashion identical to that in the AdCFTR experimental treatment period. The vehicle control period lasts 35 days. The individual must enter the vehicle control period within 1 month of completing the baseline period. If the time since ending the baseline period is greater than 7 days, there will be a reassessment of the safety and efficacy parameters as detailed in Figure 5.6.3-A.

5.6.4 AdCFTR Experimental Treatment Period

The individual will enter the treatment period within 1 month after completing the vehicle control period. Prior to the administration of the vector, there will be a reassessment of all parameters if the vehicle control period was greater than 1 week previously. The individual will then be ready for administration of the vector on day 1 of the experimental treatment period.

5.6.2-A
BASELINE PERIOD[1]

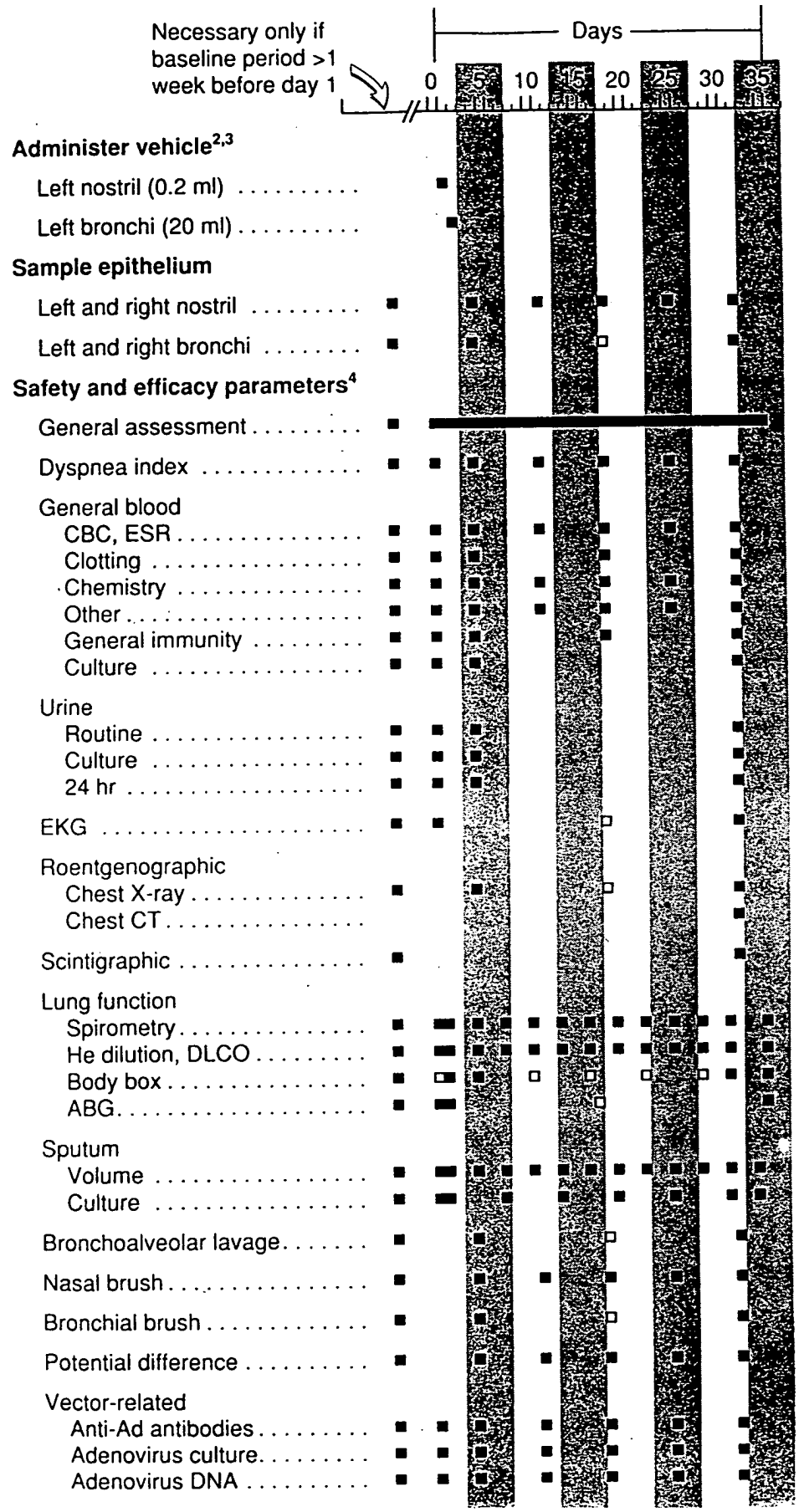


Footnotes, Figure 5.6.2-A

[1] Individuals will be evaluated in pairs. Movement from baseline period to vehicle control period will occur if all study inclusion and exclusion criteria are met at the completion of the baseline period.

[2] For definitions and details see section 5.5; ■ = test will be performed; □ = test optional.

5.6.3-A
VEHICLE CONTROL PERIOD[1]



Footnotes, Figure 5.6.3-A

[1] Movement from vehicle control to AdCFTR period will occur within one month of completion of the vehicle control period.

[2] Vehicle: 10 mM Tris-Cl, pH 7.4, 1 mM $MgCl^2$, 3.3% glycerol.

[3] Administration of vehicle will be to the left nostril and the left bronchus (with the right nostril and bronchus as controls) unless anatomic/clinical reasons suggest the opposite side is better; if so, the vehicle will be administered to the right side and the left side will be the control.

[4] For definitions and details, see section 5.5; ■ = test will be performed; □ = test optional; for general blood studies, on day 1, CBC and ESR will be sampled at 4 hr and 8 hr, on day 2, at 4 hr and 8 hr.

The 2 individuals in each treatment group will be staggered by a minimum of 1 week to insure that no serious acute adverse reactions have occurred at the dose level for that group. Assuming no adverse effects in either individual of the treated group and no adenovirus can be detected in nasal, pharynx, blood, rectal or urine cultures on 3 separate days, the protocol will move to the group receiving the next dose level no earlier than 1 week after the second individual (at the previous dose level) is treated. A similar pattern will be followed for each group.

If an intercurrent illness occurs during the therapy period, it will be managed as described in Section 5.6.5.

If an adverse event occurs during the therapy period, it will be managed as described in Section 5.6.6.

The vector will be administered at the dosage and to the sites described in Section 5.6.1. The nasal administration will be carried out through a nasal speculum under direct vision to the inferior turbinate. The bronchial administration will be carried out slowly to a main stem bronchus via a fiberoptic bronchoscope.

To minimize the risk from intercurrent infection (viral or other) during the initial phase after administration of the vector, the individual will be isolated (see above) for 10 days prior to being treated with the vector and will be clinically evaluated prior to administration of the vector to insure there is no intercurrent viral illness. Further, after administration of the vector, the patient will continue in isolation until demonstrated not to be shedding (nose, pharynx, urine, rectal) the vector or replication competent adenovirus on at least three different days.

From the data available, it is very unlikely that shedding of AdCFTR will extend beyond a few days to a few weeks (see sections 4.1, 4.5, 4.7, 4.8, 4.9). If the situation does arise that shedding continues, the patient receiving AdCFTR will remain in the Clinical Center under the conditions described in this section until shedding is no longer detected on 3 separate days. This mandatory criteria for participation in the protocol, with the caveats regarding the rights of the individual to leave, is described in the Informed Consent (see section 5.13).

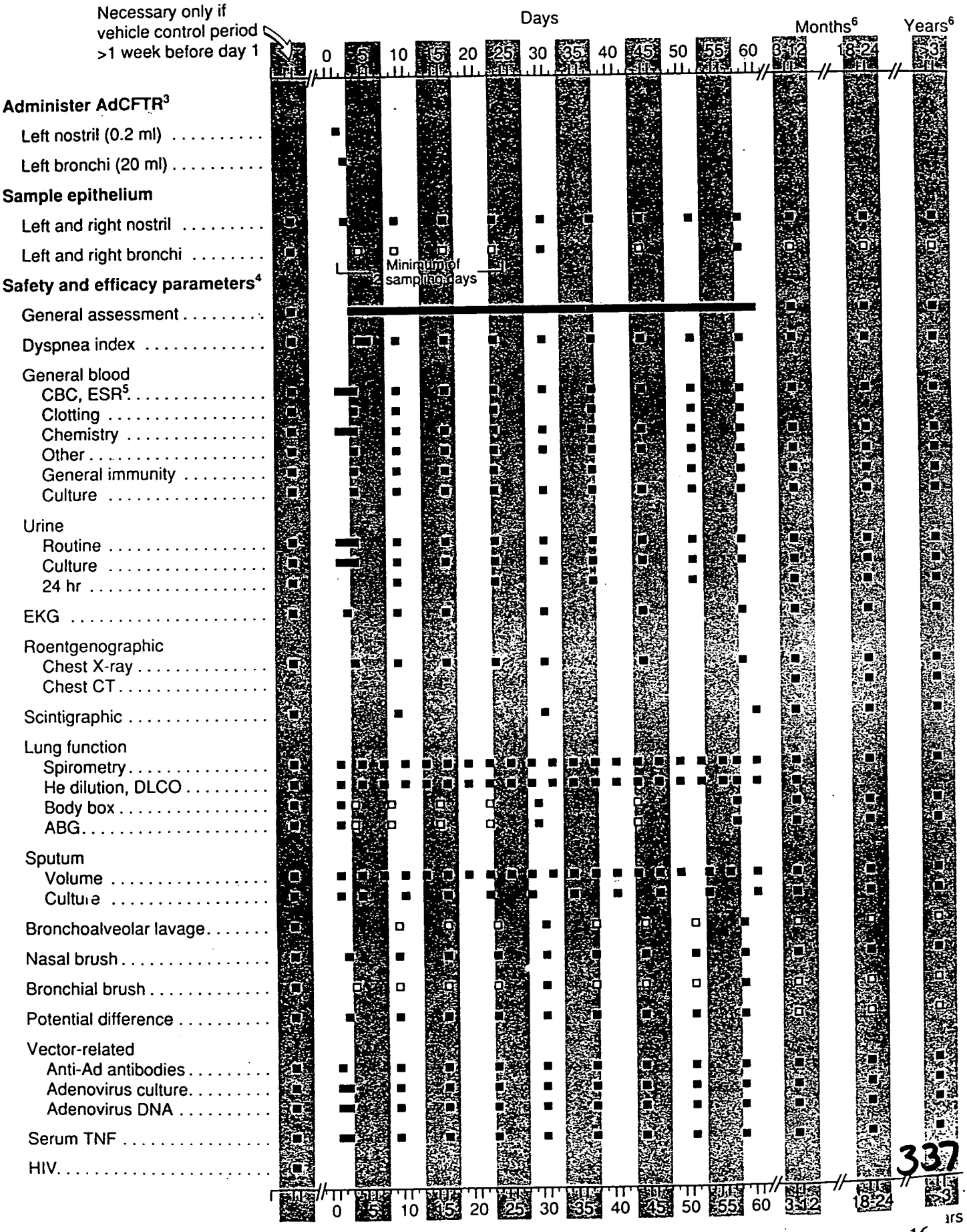
To insure protection of the environment, the patient rooms to be used on the Pulmonary Branch 7 West clinical unit, building 10 will be converted to be negative pressure to the corridor and filtered as approved by the Biosafety Office, NIH.

After administration of the vector, the patient will be evaluated with the safety and efficacy parameters as detailed on the time line in Figure 5.6.4-A.

5.6.5 Management of Intercurrent Illness

The natural history of cystic fibrosis is associated with exacerbations of the disease, particularly the respiratory manifestations (Boat et al., 1989). In this context, it is possible that among the 10 individuals in this protocol,

5.6.4-A
AdCFTR EXPERIMENTAL TREATMENT PERIOD[1,2]



Footnotes, Figure 5.6.4-A

[1] Group 1, n = 2, 10^6 pfu/ml, 0.2 ml/nostril, 20 ml/bronchus
Group 2, n = 2, 10^8 pfu/ml, 0.2 ml/nostril, 20 ml/bronchus
Group 3, n = 2, 10^9 pfu/ml, 0.2 ml/nostril, 20 ml/bronchus
Group 4, n = 2, 10^{10} pfu/ml, 0.2 ml/nostril, 20 ml/bronchus
Group 5, n = 2, 10^{11} pfu/ml, 0.2 ml/nostril, 20 ml/bronchus

[2] The n = 2 individuals in each group will be treated in parallel; subsequent groups will enter the protocol 2 weeks after administration of AdCFTR to the preceding group if no adverse reactions have been observed (see section 5.6.6) and no adenovirus can be detected in nasal, pharynx, blood, rectal or urine cultures of the preceding group on 3 separate days.

[3] Administration of AdCFTR will be to the left nostril and left bronchus (with the right nostril and bronchus as controls) unless anatomic/clinical reasons suggest the opposite side is better; if so, AdCFTR will be administered to the right side and the left side will be the control.

[4] For definitions and details, see section 5.5; ■ = test to be performed; □ = test optional.

[5] On day 1, samples at 4 hr, 8 hr; on day 2, samples at 4 hr, 8 hr.

[6] "18-24" = evaluation at months 18 and 24; "years" = yearly evaluation starting at year 3.

exacerbations of respiratory infections and/or decrement in clinical lung-related parameters will occur during the baseline period, vehicle control period, or AdCFTR experimental treatment period. Further, the natural history of the disorder suggests it is possible that exacerbations of manifestations of the disease in other organs might also occur.

Should intercurrent exacerbations of disease of any organ occur either during initial assessment or after therapy with the vector, the patients will be treated by conventional clinical therapy. During the period of intercurrent illness, assessment of safety and efficacy parameters will continue as defined by the protocol as long as such assessment does not interfere with the clinical care and welfare of the patient.

If intercurrent illness occurs during the baseline period or vehicle control period, efforts will be made to determine if adenovirus is involved by nasal, pharynx, urine and rectal culture. If adenovirus is definitively shown to be involved, the individual will be removed from the protocol and not allowed to enter the AdCFTR experimental treatment period.

If the intercurrent illness during the baseline or vehicle period control period is sufficiently severe to preclude continuation of the protocol, the individual will be removed from the protocol.

If intercurrent illness occurs during the AdCFTR experimental treatment period, efforts will be directed toward determining whether the vector or replication competent adenovirus is involved, including viral culture of nasal, pharynx, urine and rectum and assessment of respiratory epithelial cells and inflammatory cells for the presence of AdCFTR or replication competent adenovirus DNA. If the intercurrent illness (independent of course) during this period is sufficiently severe to preclude further participation, the individual will be removed from the protocol, but will remain in the Clinical Center until proven not to be shedding adenovirus as dictated by the protocol (see section 5.6.4).

If a study individual has to be removed from the protocol because of intercurrent illness, a new individual will be substituted to begin the protocol. Removal of an individual will be reported to the Institutional Clinical Research Subpanel (ICRS), NHLBI; the Institutional Biosafety Committee (IBC), the National Institutes of Health (NIH); the Recombinant DNA Advisory Committee (RAC); and the Food and Drug Administration (FDA).

5.6.6 Management of Adverse Events

Adverse reactions will be defined for each organ system based on a four grade scale (Table 5.6.6-A). If an adverse reaction occurs attributable to administration of the vector, the individual will be treated by conventional clinical therapy. Assessment of safety and efficacy parameters will continue as defined by the protocol as long as such assessment does not interfere with the clinical care and welfare of the patient. No additional patients will be treated until the data is discussed with the ICRS, NHLBI, IBC, NIH, and the FDA. The occurrence of the adverse reaction will be reported to the ICRS, IBC, RAC, and the FDA within 24 hours of its recognition.

Table 5.6.6-A
Graded Toxicity Scale To Define Adverse Reactions[1]

	CATEGORY			
'ARAMETERS	Grade 1 (Minimal)	Grade II (Moderate)	Grade III (Severe)	Grade IV (Intolerable)
EMATOLOGIC[2]				
.) WBC	2501-3000/mm³	1500-2500/mm³	1000-1500/mm³	<1000/mm³
:) Granulocytes	1251-1500/mm³	751-1250/mm³	500-750/mm³	<500/mm³
) Platelets	75,001-100,000/mm³	50,001-75,000/mm³	25,000-50,000/mm³	<25,000/mm³
OAGULATION[3]				
Prothrombin time	14-16 sec	17-18 sec	>19 sec without bleeding	>19 sec with bleeding
Partial thromboplastin time	40-45 sec	46-50 sec	>51 sec without bleeding	>51 sec with bleeding
EPATIC[4]				
) Bilirubin	1.5-2.0 mg percent	2.1-2.5 mg percent	2.6-3.0 mg percent	>3.0 mg percent
.) SGOT	50-150 IU	151-300 IU	301-600 IU	>600 IU
ENAL				
.) BUN	25-50 mg percent	51-75 mg	76-100 mg percent	>100 mg percent
:) Creatinine	2.0-2.5 mg percent	2.6-3.0 mg percent	3.1-3.5 mg percent	>3.5 mg percent
ASTROINTESTINAL				
) Nausea/vomiting	Nausea alone	≤5 emeses/day	>5 emeses/day	Intractable vomiting/needs IV
) Diarrhea[5]	3-4 loose stools a day	5-7 loose to watery stools a day	>7 stools a day	Bloody diarrhea or needs IV fluids
) Anorexia/ weight loss	Anorexia without weight loss	<5 percent weight loss	5-10 percent weight loss	>10 percent weight loss
) Stomatitis	Sore mouth	≤3 discrete ulcers	>3 discrete ulcers	>3 discrete ulcers unable to eat
ATIGUE	Fatigue without decrease in activities of daily living	In bed ≤50 percent of the day	In bed > 50 percent of the day	Unable to care for self
EUROLOGIC				
) Peripheral	Transient (≤12 hrs) numbness or paresthesia	Persistent (>12 hrs) numbness or paresthesia	Decrease of deep tendon reflexes	Motor weakness or autonomic dysfunction
) Central	Mild difficulty with concentration	Moderate difficulty with concentration	Confusion disorientation	Coma, seizures
EVER[2,6]	T 38.0-39.0°C	T 39.0-40°C	T 40-42°C	T >42°C
YPOTENSION	20-25 percent drop from baseline systolic	26-30 percent drop from baseline systolic	31-40 percent drop from baseline systolic	>40 percent drop from baseline systolic, symptomatic
IGORS	Not requiring medication	occurring with the use of medication	symptomatic hypotension, acrocysnosis, severe tachyprise	rigors associated with Grade III toxicity hypotension requiring medication
ULMONARY[7]	15-20 percent decrease from baseline	21-30 percent decrease from baseline	31-50 percent decrease from baseline	>50 percent decrease from baseline
RONCHIAL	Visible hemorrhage	visible hemorrhage/ ulceration	active ulceration/ ulceration/ increased edema	massive bleeding and edema

Footnotes, Table 5.6.6-A

[1] During the AdCFTR experimental therapy period, all adverse reactions will be reported along with the clinical and laboratory assessment as to whether the adverse reaction is associated with the AdCFTR vector, the procedure used to assess safety or efficacy, or an intercurrent illness.

[2] Marked increase in white blood counts with an increase in percent neutrophils is frequent in individuals with cystic fibrosis following bronchoscopy. Fever of 39-40° is also frequent.

[3] Despite vitamin K therapy

[4] Above baseline

[5] Above baseline for grades I - III

[6] For greater than 6 hours despite antipyretic therapy

[7] Lung function tests, including forced vital capacity and forced expiratory volume in 1 second

If an adverse event occurs in response to the obtaining of safety and efficacy parameters unrelated to the vector itself, the assessment of safety and efficacy parameters will continue as defined by the protocol as long as such assessment does not interfere with the clinical care and welfare of the patient.

If a study individual has to be removed from the protocol because of an adverse reaction unrelated to the AdCFTR vector, a new individual will be substituted to begin the protocol. This will be reported to the ICRS, NHLBI, IBC, NIH, RAC and the FDA. If the study individual has to be removed from the protocol because of an adverse relation related to the AdCFTR vector, a decision to add a substitute patient will be made only after discussion and agreement of the ICRS, NHLBI, IBC, NIH, and the FDA.

Should the patient die while in this protocol, the family will be asked to give permission for a full autopsy to determine the precise cause of death (see Consent form). Samples of all tissues obtained at autopsy will be evaluated for the presence of AdCFTR and replication competent Ad5 using PCR and viral cultures as outlined in the protocol.

5.6.7 Ability of the Protocol to Answer the Questions Posed in the Introduction to the General Design (Section 5.1)

As detailed in Section 5.1, the protocol is designed to answer six questions central to evaluating the use of a replication deficient, recombinant adenovirus containing the human CFTR cDNA to treat the respiratory manifestations of cystic fibrosis. The following details how the protocol design will answer these questions. Conclusions regarding the answer to each question will be made by statistical evaluation appropriate for each parameter. At a minimum, however, a formal data base will be established for all parameters measured.

(1) Is it safe? The extensive general and adenovirus-related safety data will answer this question for a one time administration to the target organs as a function of increasing dose.

(2) Will it provide biologic correction in respiratory epithelial cells? This question will be answered by assessing respiratory epithelial cells before and after therapy for a variety of biologic parameters relevant to expression of the CFTR gene.

(3) Chronicity? If biologic correction is achieved, the protocol will define how long the correction will last in the target organ.

(4) Respiratory epithelium electrical potential difference correction? Based on the knowledge that individuals with cystic fibrosis have an elevated potential difference between the epithelial surface and the subcutaneous tissues secondary to the primary genetic abnormality, the protocol will determine whether the therapy will correct the epithelium in vivo.

(5) Clinical improvement? A variety of lung-related clinical parameters will be used to assess improvement relevant to the clinical status of the patient.

(6) Does humoral immunity develop against the vector and, if so, will it prevent chronic administration in the future? From the in vivo data generated in experimental animals and the in vitro data showing correction of the CF phenotype in epithelial cells derived from individuals with CF, it is very likely that respiratory administration of the adenovirus vector containing the normal human CFTR cDNA should correct the CF phenotype at the biologic level. While it is likely that the correction may persist for a significant period, the fact that the adenovirus does not transfer genes to the genome of most target cells, and the normal turnover (albeit slow) of the respiratory epithelium, suggest that therapy will have to be periodic. In this case, it is an important goal of this protocol to define the humoral immune response to respiratory administration of the vector. This will be defined in the serum and lung epithelial lining fluid by periodic measurements. If anti-vector antibodies are detected, they will be evaluated for their neutralizing activity (if any) in regards to the ability of the vector to infect and transfer the CFTR cDNA to human epithelial cell lines and freshly isolated human respiratory airway epithelial cells. This will be evaluated with serum and epithelial lining fluid sampled over time after the administration of the vector.

In the design of this protocol it is recognized that with the limited number of individuals to be studied (n=10) and/or the doses used for only n=2 at each dose, that no biologic efficacy may be detected. If no biologic efficacy is detected and there are no safety problems associated with the AdCFTR vector, the approving committees will be asked to allow the number of study individuals to be increased.

5.8 Additional Safety Issues

5.8.1 Health Care Workers

During the baseline and vehicle control periods, there are no additional safety issues for health care workers beyond those for usual clinical procedures for the evaluation and care of individuals with cystic fibrosis. During the AdCFTR experimental therapy period, the health care workers will be exposed to no additional safety concerns beyond those for dealing with patients with infectious virus infection that can be spread by the respiratory and/or oral-fecal routes. Recommendations of the NIH Biosafety Office will be followed for handling patients, biologic materials, bedding, towels, etc. Appropriate training sessions developed by the Pulmonary Branch, NHLBI in conjunction with the NIH Biosafety Office will be used to educate all health care workers as to the protocol and relevant hazards, precautions and procedures.

5.8.2 Environment

After receiving the AdCFTR experimental treatment, the risk to the environment while the patient is on the 7 West ward will be negligible, based on the isolation procedures and patient rooms as described in section 5.6.4. In the context that the patient will not be discharged until shedding from blood, urine, nose, pharynx, or rectal sites has been documented not to occur on three separate days, the risk to the environment after discharge is limited to the possibility that the individual will become infected with a virus that will either complement or recombine with AdCFTR DNA still present in this individual. From the data available (see sections 4.1, 4.5, 4.7, 4.8, 4.9 and Table 5.2-A), this is very unlikely, but cannot be proven impossible without carrying out the human trial.

If complementation were to occur, the risk to the environment might be small amounts of AdCFTR released. Since AdCFTR is replication deficient, this will have limited spread. If an individual (other than the original patient) were to be exposed, in the context that animal studies show no risk from the exogenous gene (the CFTR cDNA), there should be no risk, particularly in the context that shedding will be associated with far less amounts of AdCFTR that will be administrated to the study individuals.

If recombination were to occur, the most likely possibility is that it would occur from a crossover between the left end of the new infecting virus (providing the missing E1 functions) and the right end of AdCFTR i.e., (this would produce a replication competent $E3^-$ Ad5). As discussed in section 4.8, this should be no different, and likely less virulent, than a replication competent $E3^+$ adenovirus. This concept is supported by a human trial with oral administration of a replication competent $E3^-$ recombinant hepatitis adenovirus vaccine (Tacket et al., 1992).

In the event that AdCFTR continues to be shed from the study individual following administration, or AdCFTR recombines with another virus or other genetic information to create a new replication competent virus that continues to be shed by the study individual, it is theoretically possible that either AdCFTR or a new replication competent virus could be released to the environment. Together, the available data argues that this theoretical possibility does not pose a risk to the environment should it occur. The unlikely scenario of the study individual insisting on leaving the isolation conditions with the knowledge of shedding of either AdCFTR or a new virus is discussed in detail in section 5.2 and in the Consent Form (section 5.13), as are the possible recourses of the physicians caring for the individual if they felt the study individual posed a risk for society.

5.9 Risk-Benefit Considerations for the Patient

The risks to the patient, and the efforts made to minimize those risks, are detailed in sections 5.1-5.6. Cystic fibrosis is a fatal disease with no alternative therapies other than transplantation, a high risk, unproven and costly procedure (see sections 1.1-1.5). The definite benefits of this initial toxicity/efficacy study are minimal, although there is a possibility that the AdCFTR experimental treatment will stabilize the respiratory disease, at least in one lung. These risk-benefit considerations are detailed for the patient in the Informed Consent.

5.10 Post-Study Patient Follow-up

The detailed data for the study will accrue during the baseline period, the vehicle control period and the AdCFTR experimental treatment period through day 60. Following discharge (see section 5.6.4 for criteria), there will be follow-up monthly for months 5-12, every 6 months for months 18 and 24, and thereafter on a yearly basis.

5.11 Clinical Facilities for the Study

The study will be conducted on the Pulmonary Branch, NHLBI 7 West in-patient care unit in the NIH Clinical Center. The patient rooms to be used will be devoted entirely to this study and will be converted to negative pressure to the corridor and filtered as approved by the Biosafety Office, NIH. The 7 West in-patient care unit is appropriately equipped to handle all relevant inter-current illnesses and adverse reactions and the Pulmonary Branch physicians are fully qualified to handle these patients and relevant possible complications. Following discharge, follow-up evaluations of the patients will be in regular patient rooms on the NHLBI 7 West in-patient care unit or in the NHLBI Outpatient Clinic, ACRF.

5.12 Privacy and Confidentiality

The privacy of the patients and all confidentiality issues will be handled in accordance with the NIH Clinical Center guidelines

5.13 Informed Consent

The informed consent document that follows is in the format required by the NIH and is found at the end of section, after 5.15.

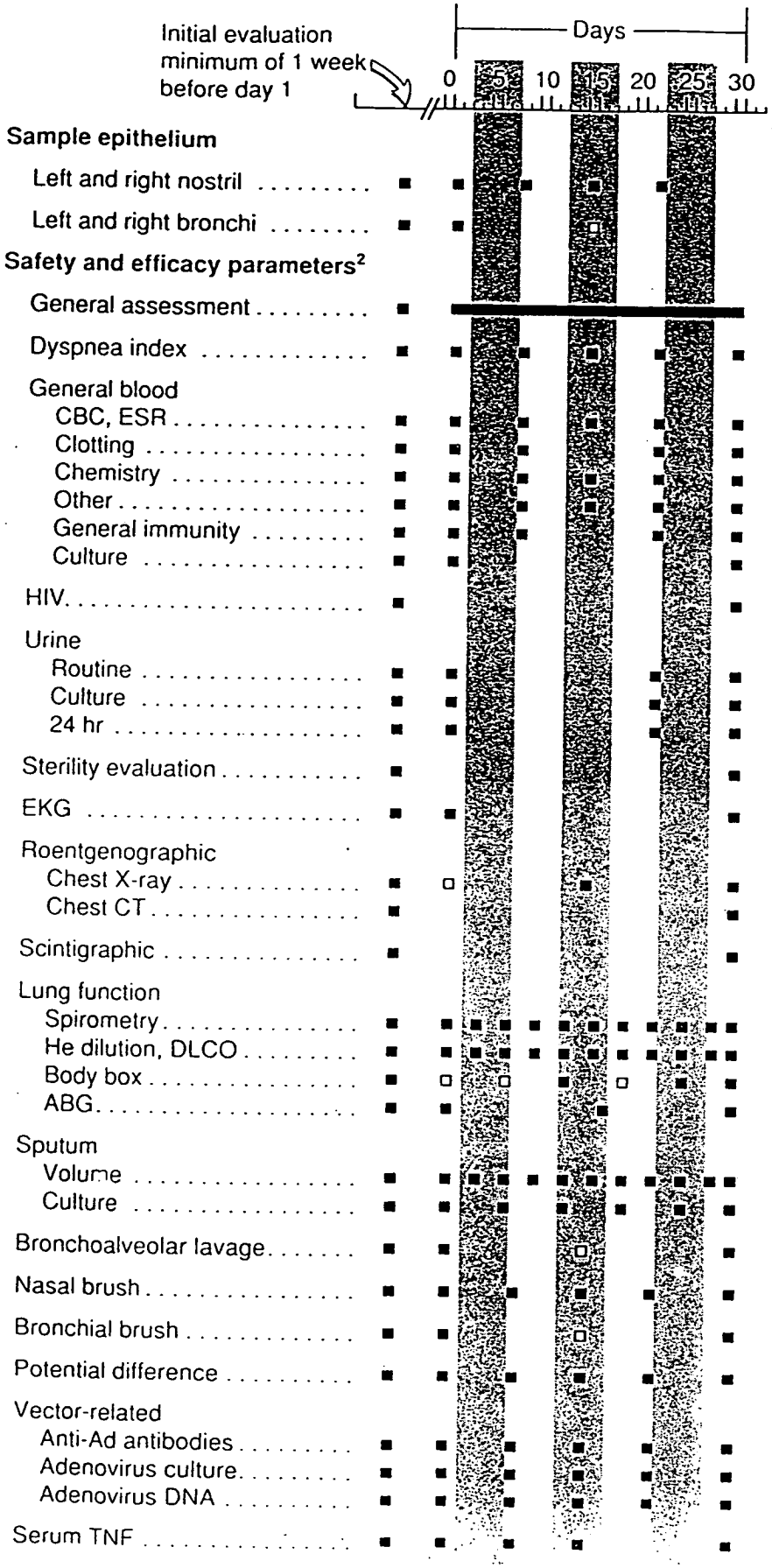
5.14 Reporting of Serious Adverse Effects

As detailed in section 5.6.6, reports of adverse reactions will be made to the ICRS, NHLBI, IBC, NIH, RAC, and the FDA within 24 hours of its recognition in accordance with the guidelines of each of these groups.

5.15 Future Directions

Based on this initial safety and efficacy data, future studies will be designed regarding dose, frequency of administration and safety and efficacy parameters. Appropriate statistical and data base considerations will be determined in consultation with the Biostatistics Research Branch, NHLBI.

5.6.2-A
BASELINE PERIOD[1]

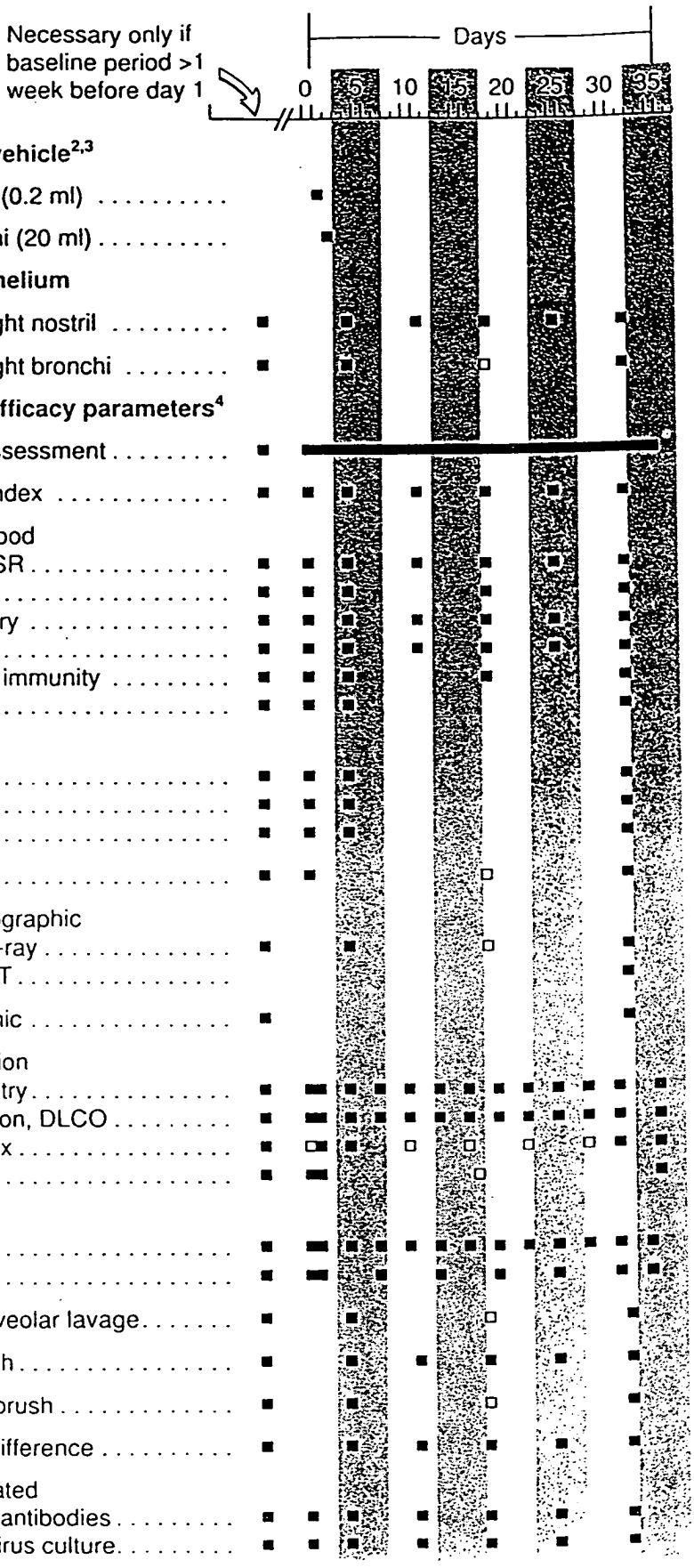


Footnotes, Figure 5.6.2-A

[1] Individuals will be evaluated in pairs. Movement from baseline period to vehicle control period will occur if all study inclusion and exclusion criteria are met at the completion of the baseline period.

[2] For definitions and details see section 5.5; ■ = test will be performed; □ = test optional.

5.6.3-A
VEHICLE CONTROL PERIOD[1]



Footnotes, Figure 5.6.3-A

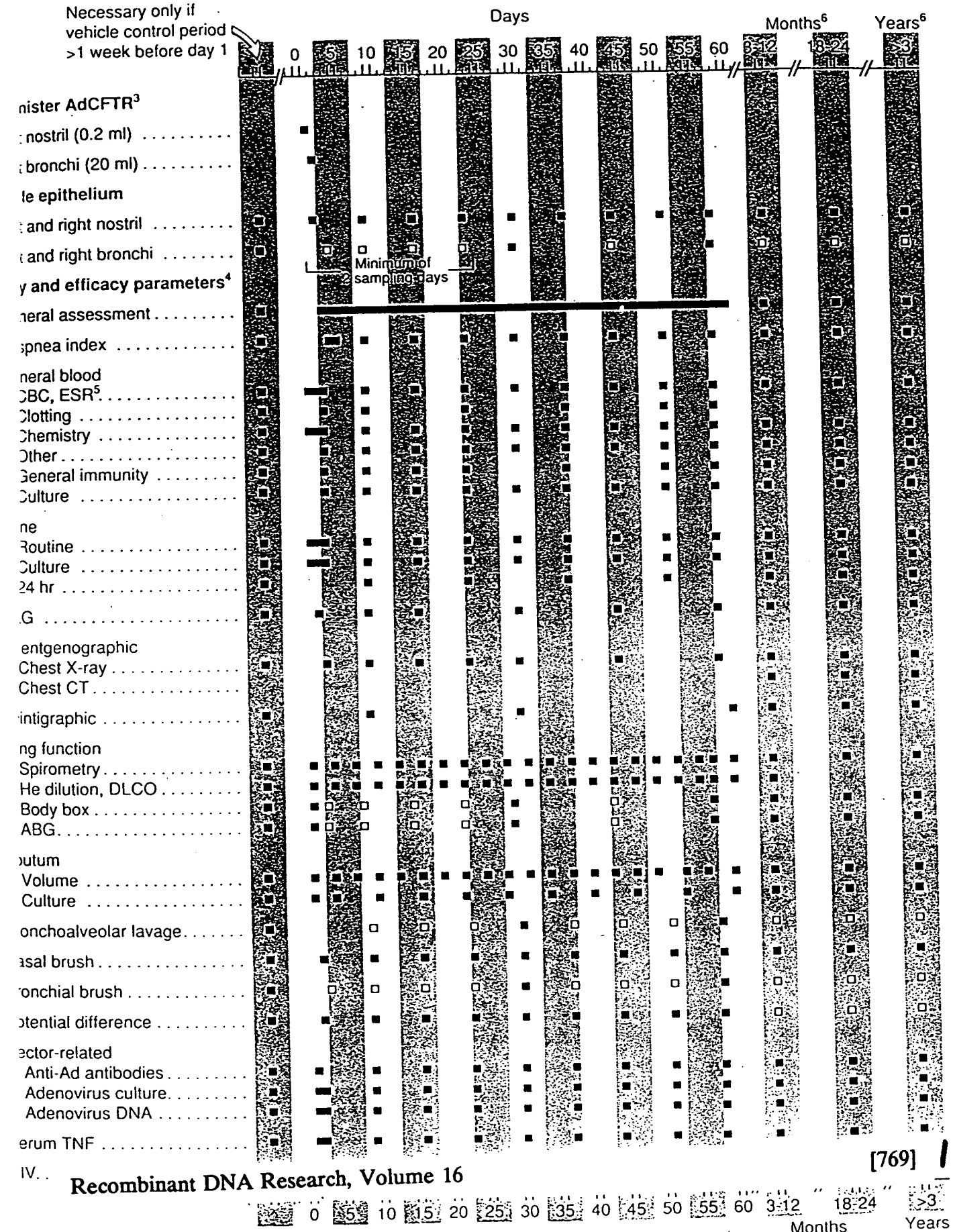
[1] Movement from vehicle control to AdCFTR period will occur within one month of completion of the vehicle control period.

[2] Vehicle: 10 mM Tris-Cl, pH 7.4, 1 mM $MgCl^2$, 3.3% glycerol.

[3] Administration of vehicle will be to the left nostril and the left bronchus (with the right nostril and bronchus as controls) unless anatomic/clinical reasons suggest the opposite side is better; if so, the vehicle will be administered to the right side and the left side will be the control.

[4] For definitions and details, see section 5.5; ■ = test will be performed; □ = test optional; for general blood studies, on day 1, CBC and ESR will be sampled at 4 hr and 8 hr, on day 2, at 4 hr and 8 hr.

5.6.4-A
AdCFTR EXPERIMENTAL TREATMENT PERIOD[1,2]



Footnotes, Figure 5.6.4-A

[1] Group 1, n = 2, 10^6 pfu/ml, 0.2 ml/nostril, 20 ml/bronchus
Group 2, n = 2, 10^8 pfu/ml, 0.2 ml/nostril, 20 ml/bronchus
Group 3, n = 2, 10^9 pfu/ml, 0.2 ml/nostril, 20 ml/bronchus
Group 4, n = 2, 10^{10} pfu/ml, 0.2 ml/nostril, 20 ml/bronchus
Group 5, n = 2, 10^{11} pfu/ml, 0.2 ml/nostril, 20 ml/bronchus

[2] The n = 2 individuals in each group will be treated in parallel; subsequent groups will enter the protocol 2 weeks after administration of AdCFTR to the preceding group if no adverse reactions have been observed (see section 5.6.6) and no adenovirus can be detected in nasal, pharynx, blood, rectal or urine cultures of the preceding group on 3 separate days.

[3] Administration of AdCFTR will be to the left nostril and left bronchus (with the right nostril and bronchus as controls) unless anatomic/clinical reasons suggest the opposite side is better; if so, AdCFTR will be administered to the right side and the left side will be the control.

[4] For definitions and details, see section 5.5; ■ = test to be performed; □ = test optional.

[5] On day 1, samples at 4 hr, 8 hr; on day 2, samples at 4 hr, 8 hr.

[6] "18-24" = evaluation at months 18 and 24; "years" = yearly evaluation starting at year 3.